The Role of Zap70 in Thymocyte Development

Charles Sinclair

UCL

A thesis presented for the degree of Doctor of Philosophy, 2010

Statement of Declaration

I, Charles Sinclair confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Publications Arising from this Thesis

Saini, M.*, Sinclair, C.*, Marshall, D., Tolaini, M., Sakaguchi, S. & Seddon, B. (2010). Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds. *Science Signaling* **3**, ra23. *Contributed equally. (see **Appendix**).

White, A.J., Nakamura, K., Jenkinson, W.E., Saini, M., Sinclair, C., Seddon, B., Narendran, P., Pfeffer, K., Nitta, T., Takahama, Y., Caamano, J.H., Lane, P.J.L., Jenkinson, E.J., Anderson, G. Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells. *The Journal of Immunology* **185**, 4769-76.

Sinclair, C.*, Saini, M.*, Hogan, T., Sakaguchi, S., Seddon, B. Positive selection links IL-7 and antigen receptor survival signaling in T lymphocytes (Manuscript submitted). *Contributed equally.

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Ben Seddon for giving me the opportunity to work on this project, despite my minimal previous lab experience, and for the help and support throughout my time at the NIMR. Also to Dr. Andrea White and Prof. Graham Anderson, for the successful collaboration examining the temporal regulation of mTEC development, and for performing the confocal microscopy on TetZap70 thymic sections. In addition, I would like to give my appreciation to Jara Joedicke, for setting up and performing chemotaxis assays with TetZap70 thymocytes. Thanks also go to my second supervisor Dr. Alexandre Potocnik and thesis committee members Dr. Victor Tybulewicz and Dr. George Kassiotis for their critical advice and support throughout my time at the NIMR.

Thanks for the fun lab times, to the old lab: Claire Pearson, Georgina Cornish, Moni Saini, Pepa Ganchevska and Roslyn Kemp… and new lab: Thea Hogan, Ana Silva, Eve Hornsby, Sim Tung, Dan Marshall and Ina Schim van der Loeff.

Finally I would like to thank my family for their love, support, and much encouragement throughout 24 years of studying.

Abstract

CD4+CD8+ double positive (DP) precursors undergo positive selection in the thymus, and subsequently commit to either the CD4 or CD8 single positive (SP) lineage. Mice lacking Zap70, a crucial kinase involved in T-cell receptor (TCR) signal transduction are developmentally arrested at the DP stage, showing that this process depends on TCR signalling. Furthermore, the resultant SP populations are major histocompatibility (MHC) matched, as CD4 lineage cells recognise MHC-II whereas CD8 lineage T cells recognise MHC-I.

There are currently two favoured models describing how the MHC restriction of the TCR correlates with lineage choice. These suggest that differences in either the TCR signalling strength or signalling length underlie the CD4/CD8 lineage decision during the DP stage. Both models posit that differences in signalling strength/length are properties that are conferred by the different signalling capacities of the CD4/CD8 coreceptors. However, questions remain over the mechanisms behind this process, including how the selecting cell interprets the proximal differences in TCR activation and whether the quality of this signal impacts on its future homeostatic survival potential. A major obstacle in addressing these questions is the lack of tools to facilitate understanding of the kinetic regulation of positive selection. We therefore sought to examine the kinetics of T-cell development using a tetracycline inducible Zap70 mouse model (TetZap70 hereon).

In the absence of the tetracycline derivative doxycycline (dox), T-cells were arrested at the DP stage, prior to positive selection. However, the administration of dox induced positive selection of a synchronized wave of positively selecting thymocytes, enabling the resolution of intermediate populations of positively selecting DPs. We found that CD4 and CD8 lineage development occurred with temporal distinction, from phenotypically disparate populations of DP thymocytes. Furthermore, we found that endogenous Zap70 was developmentally regulated in different DP populations of WT mice, and loss of this regulation in TetZap70 mice corresponded to an impairment in CD8 lineage generation. Thus we suggest that temporal regulation of T-cell signalling sensitivity during thymic development facilitates the resolution of strong/consistent signals versus weak/intermittent signals. Finally we find evidence that the quality of the positive selection signal not only controls the CD4/CD8 lineage decision, but also impacts on the future homeostatic survival potential of T-cells by influencing levels of the prosurvival interleukin-7 receptor alpha chain (IL7rα).

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2ME	β-mercaptoethanol
ActD	Actinomycin D
ADA	Adenosine deaminase
AIDS	Acquired immundeficiency syndrome
AIRE	Autoimmune regulator
AMPS	Ammonium persulphate
AP-1	Activator protein-1
APC	Allophycocyanin
APC-A647	Allophycocyanin-Alexa-Fluor 647
APC/s	Antigen presenting cell/s
β2m	β2-microglobulin
BAD	Bcl-xL/Bcl-2-associated death promotor
BAF	Brg/Brahma-associated factors
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor

Bcl2	B cell leukaemia/lymphoma 2
BCR	B-cell receptor
BM	Bone marrow
BrdU	5-Bromodeoxyuridine
BSA	Bovine serum albumin
CamK	Calcium/calmodulin-dependent protein kinase
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
c-Cbl	E3 ubiquitin-protein ligase Cbl
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
cDMEM	Complete Dulbecco's modified Eagle's medium
cDNA	Complementary DNA
Chx	Cyclohexamide
CO_2	Carbon dioxide
CREB	cAMP response element-binding
CsA	Cyclosporin A
cTEC/s	Cortical thymic epithelial cells

DAG	Diacylglycerol
DC/s	Dendritic cell/s
dH_20	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
Dox	Doxycycline
DP	Double positive
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECR/s	Evolutionary conserved region/s
EDTA	Ethylenediaminetetraacetic acid
EF450	E-Fluor 450
EF780	E-Fluor 780
ER	Endoplasmic reticulum
Erk	Extracellular regulated kinase
Ets	E-twenty six
FA	Fatty acid

FACS	Fluorescence-activated cell sorting
FAM	6-carboxyfluorescien
Fc	Fragment crystallisable
Fcγ	Fc receptor γ-fragment
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Foxo1	Forkhead box O1
Foxp3	Forkhead box P3
FTOC	Fetal thymic organ culture
Fyn	Fyn proto-oncogene
Gabp	GA-binding protein
Gabpb1	GA-binding protein β-subunit 1
Gata3	GATA-binding protein 3
γc	Common γ-chain
Grb2	Growth receptor bound 2
HD	Helper-deficient
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase-1

HRP	Horseradish peroxidase
HSA	Heat stable antigen, CD24
HuCD2	Human CD2
IFNγ	Interferon-γ
I.P.	Intraperitoneal
I.V.	Intravenous
Ig	Immunoglobulin
IL	Interleukin
IL7R	Interleukin-7 receptor
IL7rα	Interleukin-7 receptor α-chain
IL7r	Interleukin-7 receptor α-chain gene
IMDM	Iscove's modified Dulbecco's medium
Indo1 AM	Indo1 acetoxymethyl ester
int	Intermediate
IP_3	Inositol triphosphate
IRES	Internal ribosomal entry site
ISP	Immature single positive

ITAM	Immunotyrosine activation motif
JAK	Janus kinase
JNK	c-jun NH2-terminal kinase
LAT	Linker of activated T-cells
Lck	Leukocyte-specific protein tyrosine kinase
LCR	Locus control region
LN	Lymph node
LNT/s	Lymph node T-cell/s
LPS	Lipopolysaccharide
LTi	Lymphoid tissue-inducer
mAb/s	Monoclonal antibody/antibodies
MAPK	Mitogen activated protein kinase
MEK	MAPK/Erk kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MTEC	Medullary thymic epithelial cell
Myc	Myc proto-oncogene protein

n/a	Not applicable
n/s	Not specified
Nck	Non-catalytic region of tyrosine kinase adaptor protein-1
NFAT	Nuclear factor of activated T-Cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NHEJ	Non-homologous end joining
NIMR	National institute for medical research
NK	Natural killer
NKT/s	Invariant natural killer T-cell/s
NP40	Nonidet P-40/Igepal ca630
NTOC/s	Neonatal thymic organ culture/s
OCT	Optimal cutting temperature
Orai	Orai calcium release-activated calcium modulator
PAMP	Pathogen-associated molecular patter
PB	Pacific blue
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis

PDK1	3-phosphoinosite-dependent protein kinase-1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PETR	PE-Texas red
PH	Plekstrin homology
PI3K	Phosphatidylinositide-3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLCγ	Phospholipase C γ-isoform
PMA	Phorbol 12-myristate 13-acetate
pMHC	Foreign peptide antigen displayed in the context of MHC
PO	Pacific orange
PRR	Pattern-recognition receptor
pSTAT	Phosphorylated signal transducer and activator of transcription
PVDF	Polyvinylidene difluoride
Raf	Raf proto-oncogene serine/threonine-protein kinase

Ras	Ras proto-oncogene
RasGRP	Ras guanyl nucleotide-exchanging protein
RSS	Recombination signal sequence
RTE/s	Recent thymic emigrant/s
RTOC	Reaggregate thymic organ culture
rtTA	Reverse tetracycline transactivator protein
Runx	Runt-related transcription factor
S1P	Sphingosine-1-phosphate
S1P1	Sphingosine-1-phosphate receptor-1
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
siRNA	Small inhibitory RNA
Slp76	SH2 domain containing leukocyte protein of 76kDa
SOCE	Store operated calcium entry
Sos	Son of sevenless
SP	Single positive

SPF	Specific pathogen free
spMHC	Self-peptide antigen displayed in the context of MHC
SSc	Side scatter
STAT	Signal transducer and activator of transcription
Stim	Stromal interaction molecule
Tap	Transporters associated with antigen processing
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TetZap70	*Zap70^{Tre} rtTA^{huCD2} Zap70$^{-/-}$*
TetZap70^{on}	Constitutively (≥7d) dox fed TetZap70 mice
T_H	T-helper
T_C	T-cytotoxic
T_{CM}	Central memory T-cell/s
T_{EM}	Effector memory T-cell/s
ThPOK	T-helper-inducing POZ/Kruppel-like factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

TOX	Thymus high-mobility box protein
TRA/s	Tissue-restricted antigen/s
TRE	Tetracycline response element
Treg/s	Regulatory T-cell/s
TSP	Thymus seeding progenitor
Vav	Vav oncogene
WT	Wild-type
Y	Tyrosine
Zap70	Zeta-chain-associated protein kinase of 70kDa molecular weight

Units used in this thesis

ºC	Degrees Celsius
%	Percentage
sd	Standard deviation
$^{+/-}$	Heterozygous gene knockout
$^{-/-}$	Homozygous gene knockout
Δ/Δ	Homozygous genetic locus deletion

bp	Base pairs
kb	Kilobase pairs
h	Hours
d	Days
μL	Microlitres
mL	Millilitres
L	Litres
nm	Nanometers
***μ*m**	Micrometres
mm	Millimetres
cm	Centimetres
kDa	Kilodaltons
***μ*g**	Micrograms
mg	Milligrams
w/v	Weight/volume
w/w	Weight/weight
v/v	Volume/volume

X	Times (magnification)
RPM	Revolutions per minute
V	Volts

Chapter 1

Introduction

1.1 The immune system

The immune system refers to integrated cellular and molecular mechanisms by which a host protects itself from pathogens. That such protective mechanisms are beneficial is highlighted by the diversity of organisms exhibiting elements of immunity. For example, bacteria possess mechanisms to prevent bacteriophage entry at both a cellular and population level (reviewed in (Labrie *et al.*, 2010)). In addition, the secretion of antimicrobial factors facilitates competition with microbial rivals (reviewed in (Hibbing *et al.*, 2010). With the development and diversification of multicellular organisms over one billion years ago (reviewed in (Kaiser, 2001)), shifting evolutionary pressure favoured the development of increasingly complex and diverse immunological mechanisms. This is typified in placental mammals, which evolved recently in the early cretaceous period (approximately 145-65 million years ago) (reviewed in (Luo, 2007)), and boast a highly complex immune system, consisting of organs, cells and molecular responses. Thus it appears to be an evolutionarily favourable trait to have resistance against disease causing organisms. Furthermore, whilst lower evolved invertebrates largely possess non-specific innate immune mechanisms, the immune system in higher evolved vertebrate species is comprised of both innate and adaptive components.

1.1.1 Innate immunity

Innate immunity refers to a non-specific response to pathogen challenge that does not confer immunological memory. At a most basic level, this can refer to physical barriers including epithelial cells that prevent access of bacteria to internal host environments. In mice and humans, barriers including skin, lung and gut epithelia represent the first line of defence against pathogens. Epithelial cells also secrete mucus containing antibacterial peptides such as defensins, further limiting the potential for microbial colonisation (reviewed in (Ganz, 2003)). Nevertheless, mechanical damage to the physical barriers can enable microbial entry. Viral or toxin-mediated injury of lung epithelia can also facillitate this process (reviewed in (Evans *et al.*, 2010). Furthermore, in an experimental system the disruption of gut epithelial membrane by administration of dextran sodium sulphate (DSS) leads to chronic colitis in mice (Okayasu *et al.*, 1990). Thus physical barriers can be overcome, either by inadvertent damage, or directed pathogenic mechanisms. Damage to epithelial barriers induces immediate and non-specific release of pro-inflammatory mediators including eicosanoids (leukotrienes and prostaglandins) and nitrous oxide (NOS) (reviewed in (Bogdan, 2001; Funk, 2001)). These pro-inflammatory molecules induce recruitment of innate immune cells to the site of infection. Subsequent microbial invasion results in the activation of a vast array of innate immune cells, which function to control the spread of infection. Innate immune cells function cooperatively with the complement system, a biochemical cascade of blood proteins that can result in agglutination, lysis or opsonisation of bacteria (reviewed in (Frank & Fries, 1991)).

Cells of the innate immune system are largely myeloid-derived including monocytes, neutrophils, macrophages, dendritic cells (DCs), mast cells, granulocytes, basophils and eosinophils. These cells largely function to phagocytose foreign microbes, or to release inflammatory mediators that augment the immune response. In addition the innate immune system also encompasses lymphoid-derived cells, such as natural killer (NK) cells. These cells are involved in tumour and viral surveillance, as well as in the recognition of invariant major histocompatibility complex (MHC) associated antigen. Such a variety of cell types underscore the variation in evasion strategies utilised by microorganisms. Although each cell type has a slightly divergent cellular role, there is also considerable redundancy in function, ensuring a robust response against pathogens.

Innate immune cells commonly recognise pathogen-associated molecular patterns (PAMPs). They do so by expressing a diverse array of germline encoded pattern-recognition receptors (PRRs). PRRs including the Lipopolysaccharide (LPS) receptor, mannose receptor, scavenger receptor and *N*-formyl-methionyl receptor aid in the binding of phagocytic immune cells (macrophages and DCs) to microbes (reviewed in (Gordon, 2002)). In addition, macrophage complement receptors are involved in the phagocytosis of opsonised pathogens (reviewed in (van Lookeren Campagne *et al.*, 2007)). Alternatively PRRs can signal to induce cellular release of proinflammatory cytokines, further upregulating an immune response. The most well-characterised of the signalling PRRs are Toll-like receptors (TLRs),

discovered in 1997 as the mammalian homologue to the Drosophila protein Toll (Medzhitov *et al.*, 1997). Subsequently, a family of 10 related TLR proteins have been identified in mice. TLR signalling leads to the activation of c-jun NH_2-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), resulting in the release of proinflammatory signalling molecules (reviewed in (Akira & Takeda, 2004; Takeda *et al.*, 2003)). However, despite the wide variety of innate cellular responses, the PAMPs recognised are limited by the diversity of germline-encoded receptors. Not all microorganisms exhibit the same common PAMPs, and thus an ability to recognise rare antigens is essential, particularly in the clearance of infections. The ability of higher organisms to alter genetic material through somatic diversification permits development of an adaptive immune response tailored to specific pathogens.

1.1.2 Adaptive immunity

Adaptive immunity refers to an antigen-specific response generated by the host, that confers long-lasting and protective immunological memory. Such a system was thought to have arisen in gnathostomes (jawed vertebrates), although the agnathan (jawless) sea lamprey also exhibits somatic diversification though a disparate mechanism (Pancer *et al.*, 2004). Thus convergent evolution of adaptive immunity highlights its importance in higher vertebrates. Adaptive immune cells are lymphoid-derived and in mice and humans consist of B and T-lymphocytes. B-cells predominately play a role in the recognition of soluble antigens (referred to as humoral immunity), whereas T-cells can recognise

either intracellular antigen or extracellular antigen taken up and presented by antigen presenting cells (APCs) (referred to as cell-mediated immunity).

Activation of B and T-lymphocytes is mediated by stimulation of polyclonal antigen receptors known as the B-cell receptor (BCR) (also referred to as the immunoglobulin (Ig) receptor) and T-cell receptor (TCR) respectively. This is a temporally late event during the course of an infection, due to an additional requirement for costimulatory signals from innate immune cells (reviewed in (Lenschow *et al.*, 1996)). Once activated lymphocytes undergo clonal expansion and effector cell differentiation. B-cells secrete a soluble form of their Ig-receptor, referred to as antibody. The binding of antibodies functions to agglutinate or neutralise soluble particles, in addition to acting as an opsonin. Phagocytes interact with the fragment-crystallisable (Fc) domain of bound antibody, thus enhancing phagocytosis (reviewed in (Daeron, 1997)). This represents an example of crosstalk between cells of the innate and adaptive immune system. In contrast to B-cells, T-cells recognise processed oligomer peptides, complexed with major histocompatibility proteins on the surface of APCs (Zinkernagel & Doherty, 1974) (reviewed in (Madden, 1995)). The cognate recognition of peptide complexed with MHC (pMHC) by the TCR, in the context of costimulation mediated by proinflammatory cytokines and costimulatory cell:cell interactions with APCs, results in T-cell activation. If the APC is infected by an intracellular pathogen T-cells exhibit cytolytic activity, thus killing the target cell (Lowin *et al.*, 1994). In contrast, activation of T-cells by 'professional APCs' can lead to cytokine production (Lassila *et al.*, 1988), and

thus modulation of the immune response (reviewed in (O'Garra, 1998)). Upon clearance of a pathogen lymphocyte effector cells largely die, although some give rise to long-lived memory cells. These memory cells are readily reactivated during a secondary immune response, conferring long lasting protection to the host (reviewed in (Freitas & Rocha, 2000)).

An essential requirement of adaptive immunity is the generation of a diverse repertoire of immune cell antigen receptors. Clonal diversification of both B-cells and T-cells occurs through the somatic recombination of antigen receptor gene loci, so that components of each antigen receptor are arranged contiguously (Brack *et al.*, 1978; Chien *et al.*, 1984). This is mediated by the V(D)J recombinase enzyme complex, consisting of recombination activating gene (Rag)1 and Rag2, which act in concert to cut antigen receptor genes between antigen receptor coding subunits and recombination signal sequences (RSS) (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). In addition, non-homologous end joining (NHEJ) components join RSS to form functioning antigen receptor chains (reviewed in (Fugmann *et al.*, 2000)). Thus, combinatorial diversity is generated through different combinations of antigen receptor gene segments. Furthermore N and P nucleotide addition at recombination sites mediates further junctional diversity (reviewed in (Schatz *et al.*, 1992)). Together, combinatorial and junctional diversity mechanisms permit the generation of a large polyclonal repertoire of lymphocyte clones, each expressing a distinct lymphocyte receptor, which potentially recognise an equally diverse range of pathogens.

The requirement for an optimally functioning adaptive immune system is highlighted in human patients lacking the *Rag1*/*Rag2* genes. These patients lack circulating T and B-cells but have normal NK-cells (reviewed in (Villa *et al.*, 2001)). This severe combined immunodeficiency (SCID) phenotype typically results in opportunistic infections during early childhood (Sadeghi-Shabestari *et al.*, 2009; Yeganeh *et al.*, 2008) (reviewed in (Sobacchi *et al.*, 2006)). Similarly *Rag1* and *Rag2* knockout mice exhibit a similar T-cell$^-$ B-cell$^-$ NK-cell$^+$ SCID phenotype (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992).

In addition to the *Rag1/Rag2* genes, a multitude of factors have been implicated in a loss of lymphocyte development and/or function (reviewed in (Fischer, 2007)). Furthermore, the dysregulation of lymphocyte homeostasis has also been linked to autoimmunity (reviewed in (Vyse & Todd, 1996)) and cancer (reviewed in (Siegel *et al.*, 2000)). Therefore, understanding the mechanisms of lymphocyte development and function in experimental systems is of clinical significance, with regards to understanding and potentially treating human disease.

1.2 T-cell biology

T-cells are constantly generated throughout life in the thymus; a specialised organ situated in the anterior superior mediastinum (Miller, 1961). The majority of T-cells express TCRα and TCRβ chains, which together form the mature TCR (Kappler *et al.*, 1983). Recognition of pMHC also depends on the presence on either CD4 or CD8, which function as TCR coreceptors (Doyle &

Strominger, 1987; Norment *et al.*, 1988). Expression of CD4 or CD8 is mutually exclusive on mature T-cells and is determined during thymic selection.

1.2.1 CD4 T-cells

CD4 lineage T-cells are referred to as T-helper (T_H) cells, as they commonly secrete cytokines that modulate the immune response upon activation. The CD4 lineage comprises $CD4^+CD8^-$ cells that specifically recognise antigen in a complex with MHC-II molecules (Doyle & Strominger, 1987). MHC-II is a dimer consisting of α and β-chains, and is expressed in 'professional APCs', which include DCs, macrophages and activated B-cells. These cells take up and degrade extracellular antigen to 10-12mer peptides in endosomal compartments (Hunt *et al.*, 1992; Lassila *et al.*, 1988). Endosomes subsequently fuse with vesicles containing empty MHC-II molecules and peptides are loaded through the action of the peptide exchange catalyst H2-M in mice (HLA-DM in humans) (reviewed in (Watts, 1997)). Thus CD4 T-cells generally recognise extracellular antigens and produce cytokines to modulate the immune response.

1.2.2 CD8 T-cells

The CD8 T-cell lineage is referred to as cytotoxic, as upon activation by pMHC these cells exhibit cytolytic activity and kill their target cell. The CD8 coreceptor confers specificity to MHC-I molecules (Norment *et al.*, 1988), which are expressed on the majority of somatic cells. MHC-I consists of an α-chain ionically bonded to a β_2-microglobulin (β_2m) subunit (reviewed in (Ploegh *et al.*, 1981)). Internal peptides are constantly turned over, through the process of

ubiquitination and proteosomal degradation. Degraded peptides are transported into the endoplasmic reticulum (ER), a process that depends on transporters associated with antigen processing (Tap)1 and Tap2 (reviewed in (York & Rock, 1996)). Here they undergo further degradation to 8-10mer peptides (Hunt *et al.*, 1992), before being loaded onto MHC-I molecules. The recognition of foreign antigen occurs when cells are infected by intracellular pathogens, and the cytotoxic activity of CD8 lineage cells results in target cell lysis. This exposes intracellular antigens to humoral or phagocytic responses and kills obligate intracellular pathogens that cannot survive outside of a host cell.

Therefore CD4 and CD8 lineage cells have different functions and recognise different types of APC. The mutually exclusive expression of the CD4 or CD8 coreceptor facilitates the unique recognition of either MHC-II or MHC-I by the corresponding lineage. Thus such a mechanism permits the independent development and function of two cellular lineages utilising the same antigen receptor.

1.3 Differentiation of mature T-cells

Following development, T-cells emigrate from the thymus and reside primarily in the lymph nodes (LN), spleen and bone marrow (BM), or recirculate in the blood (reviewed in (Butcher & Picker, 1996)). T-cells express polyclonal TCRs, conferring clonal-specific avidity for both self and foreign peptides (reviewed in (Freitas & Rocha, 2000)). A T-cell clone will undergo further differentiation upon

activation by a specific pMHC complex. Furthermore, some activated T-cells will go on to form long lived memory cells, facilitating protective immunity following antigen re-exposure. Hence, there exist multiple subsets of phenotypically distinct mature T-cells that are in disparate states of differentiation.

1.3.1 Naïve T-cells

Mature T-cells are referred to as naïve prior to stimulation with pMHC and these cells make up the majority of the peripheral T-lymphocyte pool. Naive cells are phenotypically characterised by low expression of CD44 and CD25 (reviewed in (Harty & Badovinac, 2008)). In addition, they express intermediate to high levels of the interleukin-7 receptor alpha subunit (IL7rα) (Sudo *et al.*, 1993).

1.3.2 Effector T-cells

Upon stimulation T-cells undergo clonal expansion, and differentiation to a number of different effector subsets. Effector T-cell fate depends largely on the context of activation and the number of effector subsets has been much expanded in recent years. Originally the T_H1/T_H2 paradigm was established to distinguish two functional outcomes in CD4 lineage cells (Mosmann *et al.*, 1986) (reviewed in (Mosmann & Coffman, 1989)). Activation of T-cells in the presence of interleukin (IL)4 results in T_H2 cell differentiation, a common occurrence during infection by extracellular parasites. In contrast, activation in the presence of interferon-γ (IFNγ) and IL12 results in T_H1 polarisation, which commonly occurs during bacterial infections (reviewed in (O'Garra, 1998)).

More recently, further subdivisions of T-effector populations have been described, for example the T_H17 and T_H9 effector states, based on associated cytokine secretion (IL17 and IL9 for respectively) (reviewed in (Zhu *et al.*, 2010).

In contrast, CD8 lineage cells primarily perform a cytotoxic function in the immune system. However, CD8 cells also function to produce cytokines, and can be classified in terms of their cytokine expression profiles. For example T-cytotoxic (T_C)1, T_C2 and T_C17 cell subsets have been identified, expressing T_H1, T_H2 and T_H17-associated cytokines, whilst retaining cytotoxic activity (Hamada *et al.*, 2009; Li *et al.*, 1997). Therefore, in addition to developmentally instructed differences between the CD4 and CD8 lineages, the context of the T-cell activation signal triggers a specified effector response for cells within both CD4 and CD8 T-cells. This has functional implications, as the type of effector response elicited can result in different outcomes following infection. A classic example is the finding that decreases in T_H1 polarizing cytokines underlie a genetic susceptibility to the obligate intracellular pathogen *Leishmania major*, which is thought to be cleared by a T_H1 response in mice (Guler *et al.*, 1996)). In humans, T_H1 versus T_H2 responses define the outcome of *Mycobacterium leprae* infection (reviewed in (Modlin, 1994)).

Activated T-cells are further characterised by an increase in cell size, production of large amount of cytokines and changes in surface phenotype. Cells upregulate the activation markers CD69, CD25 and CD44, whereas IL7rα expression is downregulated. Following clearance of a pathogen, up to 95% of

effector T-cells undergo apoptosis, whilst some survive to become long-lived memory cells (reviewed in (Harty & Badovinac, 2008)).

1.3.3 Memory T-cells

The generation of immunological memory is the hallmark of adaptive immunity. Memory cells are rapidly activated during the secondary immune response, if an antigenic stimulus is re-encountered. Memory cells can be subdivided into effector memory T-cells (T_{EM}) and central memory T-cells (T_{CM}). Whereas T_{EM} cells confer protective immunity, T_{CM} cells are rapidly reactive following antigenic re-stimulation, ensuring a more rapid adaptive immune response to a secondary challenge. Memory T-cells can be defined by high surface expression of CD44 and IL7rα. In addition, T_{CM} express L-selectin and C-C motif chemokine receptor (CCR)7, which are not present on T_{EM} cell subsets (reviewed in (Sallusto *et al.*, 2004; Surh & Sprent, 2008)).

Thus T-cells can potentially exist in a number of differentiated states throughout their lifespan. Whereas the naïve T-cell pool favours diversity of receptors that have the potential to recognise wide ranges of antigens, activation leads to rapid clonal expansion, effector differentiation and elimination of pathogens. The memory T-cell pool contains a restricted polyclonal repertoire, as cells have previously received antigenic stimulation. These memory cells confer protective immunity, or are poised to respond rapidly upon reinfection.

1.3.4 Regulatory T-cells

The regulatory T-cells (Tregs) comprise a T-cell sub-lineage, that functions to suppress immune responses against both self and foreign antigens, thus maintaining T-cells in a non-activated state (Sakaguchi *et al.*, 1985) (reviewed in (Sakaguchi, 2004)). These cells can either develop intrathymically, or arise during the activation of naive T-cells (reviewed in (Sakaguchi, 2004)). The absence of Treg cells results in severe multi-organ autoimmune disease in both humans and mice (reviewed in (Wildin *et al.*, 2002)). Murine Tregs constitutively express the lineage defining transcription factor Forkhead box P3 (Foxp3) (Hori *et al.*, 2003), and also express high levels of CD25 (Sakaguchi *et al.*, 1995). Conditional deletion of Foxp3 in mature Treg cells using cre-lox technology ablated their suppressive function, showing that constitutive expression of Foxp3 is required for maintenance of Treg function (Williams & Rudensky, 2007). Thus Treg differentiation represents a control mechanism whereby an excessive immune response can be dampened. Critically, the various T-cell pools are subject to additional homeostatic constraints to ensure they are optimized for their divergent functions, within the limited physical constraints of the host body.

1.4 T-cell homeostasis

Metazoan tissues and organs remain stable and constant in size, but consist of cells that constantly turnover. Older differentiated cells are lost by apoptosis and are replaced by cell division or stem cell differentiation. The various inputs

and outputs to a pool of cells depend on environmental factors. For example, during wound healing cellular input outpaces output (reviewed in (Pellettieri & Sanchez Alvarado, 2007)). Similarly, T-lymphocyte cell numbers are also maintained at a constant size throughout life, despite their constant turnover. As T-cells are dispersed in the circulatory and lymphatic systems, mechanisms have evolved to sense the surrounding space and to keep cell numbers constant. The disruption of the homeostatic control mechanisms are associated with immunopathology, including but not limited to SCID (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995), arthritis (Sakaguchi *et al.*, 2003) and cancer (Bain *et al.*, 1997). Thus maintenance the T-cell population size and cell density is essential to maintain the health of a host.

1.4.1 How do T-cells sense space?

Lymphocyte homeostasis depends on the integration of signals from a multitude of soluble survival factors in addition to signals mediated by cell:cell contact. The frequency and intensity of these homeostatic survival signals can result various homeostatic outcomes (reviewed in (Freitas & Rocha, 2000)). If cell numbers are low, there is less competition for homeostatic survival factors, resulting in increased homeostatic signalling. In contrast, if cell numbers are high, homeostatic survival factors become rate-limiting, thus limiting lymphocyte population size (reviewed in (Seddon & Zamoyska, 2003)).

This is the case for B-cells, which require survival signals mediated by both the BCR and soluble B-cell activating factor (BAFF) (reviewed in (Stadanlick &

Cancro, 2008). In contrast, for both naïve and memory T-lymphocytes, the required soluble factors include common gamma chain (γc) (CD132) cytokine signals mediated predominantly by IL7, whereas cell:cell survival signals are transduced through the TCR. Whereas pMHC complexes result in the activation of T-cells, homeostatic TCR survival signalling is mediated by weaker recognition of self-peptide antigen displayed in the context of MHC (spMHC) (reviewed in (Jameson, 2002)).

1.4.1.1 IL7 signalling

An overview of IL7-mediated signalling pathways is shown in **Figure 1.1**. IL7 was first identified as a 25 kilodalton (kDa) growth factor that could promote growth of murine B-cell precursors *in vitro* (Namen *et al.*, 1988b). IL7 mRNA was identified in the thymus (Namen *et al.*, 1988a), and recombinant IL7 was subsequently found to promote growth and survival of thymocytes (Murray *et al.*, 1989). Depletion of IL7 or blocking IL7-receptor (IL7R) activity through injection of neutralising monoclonal antibodies (mAbs) severely reduces thymic and peripheral T-cell numbers (Grabstein *et al.*, 1993; Sudo *et al.*, 1993). Furthermore, the generation of IL7 and IL7rα knockout mice confirmed the essential role of IL7 in both T-cell and B-cell development (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995). In addition, *IL7* gene transcripts have been identified in a variety of lymphoid tissues (reviewed in (Hofmeister *et al.*, 1999)), and it is appreciated that IL7 is also required for mature T-cell survival (Schluns *et al.*, 2000).

Stimulation by IL7 induces the heterodimerization of the γc and the IL7rα chain (Kondo *et al.*, 1994; Noguchi *et al.*, 1993). Janus kinase (JAK)3 is constitutively associated with the γc, whereas JAK1 is constitutively associated with IL7rα (reviewed in (Jiang *et al.*, 2005)). Heterodimerization brings JAK1 and JAK3 in close proximity with one another, resulting in their mutual phosphorylation (Foxwell *et al.*, 1995). The essential role of JAKs in transducing γc cytokine signals is evidenced in the JAK3-deficient mice, which exhibit a similar SCID phenotype to the γc-deficient mice (Cao *et al.*, 1995; DiSanto *et al.*, 1995; Nosaka *et al.*, 1995; Park *et al.*, 1995). Activated JAK1 and JAK3 phosphorylate the IL7R, providing docking sites for Signal transducer and activator of transcription (STAT) proteins (reviewed in (Jiang *et al.*, 2005)).

The predominant STAT activated by the IL7R is STAT5, which is encoded by two genes: *Stat5a* and *Stat5b* (Johnston *et al.*, 1995; Lin *et al.*, 1995). These isoforms are thought to play redundant roles within the lymphoid lineage, as a deficiency in either gene does not block lymphocyte development (Imada *et al.*, 1998; Nakajima *et al.*, 1997). In contrast, mice deficient in both STAT5a and STAT5b proteins exhibit a T-cell lymphopenic phenotype, showing that STAT5 signals are essential for lymphocyte homeostasis (Yao *et al.*, 2006). Indeed a primary function of STAT5 signalling is the upregulation of pro-survival molecules including B-cell leukaemia/lymphoma 2 (Bcl2) (Jiang *et al.*, 2004; von Freeden-Jeffry *et al.*, 1997). Furthermore, STAT5 has been implicated in the transcription of genes involved in cell cycle entry proteins including *CyclinD1*

(encoding cyclin D) and Myc proto-oncogene protein (*Myc*, encoding c-Myc) (reviewed in (Jiang *et al.*, 2005)).

Phenotypic differences in the composition of T-cell progenitor populations in STAT5a/STAT5b-deficient mice compared to IL7rα-deficient mice led authors to conclude that IL7 also signals though additional non-redundant pathways (Yao *et al.*, 2006). This conclusion is supported by the findings that in addition to STATs, IL7R activates the phosphatidylinositide-3-kinase (PI3K) pathway (Dadi *et al.*, 1994; Dadi & Roifman, 1993). The p85 α-subunit of PI3K bound to phosphorylated tyrosine (Y)449 of the IL7rα chain via its Src homology 2 (SH2) domain, and subsequently led to activation of the PI3K catalytic subunit (Venkitaraman & Cowling, 1994). PI3K can activate Akt, and this signalling pathway has been suggested to facillitate cell cycle entry, and lead to inactivation of the pro-apoptotic molecule Bcl-xL/Bcl-2-associated death promotor (BAD) (reviewed in (Jiang *et al.*, 2005)). However, mice deficient in PI3K p85 α-subunit show no defect in T-cell development or proliferation (Fruman *et al.*, 1999). Thus the physiological role of PI3K activation by IL7 remains unclear and highlights the lack of understanding of the precise downstream targets of IL7 signalling.

1.4.1.2 TCR signalling

A schematic overview of TCR signalling is shown in **Figure 1.3**. The TCR is composed of heterodimerized α and β-chains and recognises antigen complexed with MHC molecules. TCR signalling is conferred by two associated

CD3 molecules, which form a complex consisting of the αβ-TCR, CD3γε and CD3δε heterodimers and a CD3ζ homodimer. Mice deficient in any of these subunits show perturbed αβ-TCR signalling and fail to develop mature αβ-T-cells (Dave *et al.*, 1998b; Haks *et al.*, 1998; Liu *et al.*, 1993; Malissen *et al.*, 1995), showing their important role in TCR assembly and/or signal transduction. The presence of either the CD4 or CD8 coreceptor is also essential for TCR signalling. The extracellular domains of the CD4/CD8 coreceptors interact with invariant regions on the MHC-II/MHC-I molecules, conferring TCR:ligand specificity for the CD4 and CD8 SP lineages (Doyle & Strominger, 1987; Norment *et al.*, 1988). In addition, the cytoplasmic tails of CD4/CD8 associate with the Src family kinase Leukocyte-specific protein tyrosine kinase (Lck), thus playing a role in its recruitment to the TCR signalling complex (Rudd *et al.*, 1988; Veillette *et al.*, 1988). It is believed that ligand binding to the TCR induces conformational changes to the TCR/CD3 signalling complex facilitating the phosphorylation of immunotyrosine activation motifs (ITAMS) on the CD3 molecules by Lck, or by a related and partially redundant kinase Fyn proto-oncogene (Fyn) (Nika *et al.*, 2010) (reviewed in (Smith-Garvin *et al.*, 2009)).

The phosphorylated ITAMs provide docking sites for SH2-domain containing proteins, of which the Zeta-chain-associated protein kinase of 70kDa molecular (Zap70) is essential for TCR signalling (Arpaia *et al.*, 1994; Chan *et al.*, 1994a; Elder *et al.*, 1994; Negishi *et al.*, 1995). Zap70 is a Syk family kinase containing two N-terminal SH2 domains and a C-terminal kinase domain (**Figure 1.2**)

(Chan *et al.*, 1992). The SH2 domains bind to phosphorylated ITAMs on CD3ε and ζ-chains (Wange *et al.*, 1993). It is hypothesised that Zap70 must be phosphorylated on Y315 and Y319 to facillitate efficient binding to ITAMs (Deindl *et al.*, 2007), whilst the phosphorylation of Y292 is inhibitory for Zap70 function (Kong *et al.*, 1996). Furthermore, optimal Zap70 function is achieved by the additional phosphorylation of Y492 and Y493, located on the activation loop of the Zap70 kinase domain. It is believed that tyrosine phosphorylation of Zap70 is mediated by Lck or by transautophosphorylation by other Zap70 molecules (thus Y292 represents a negative feedback inhibitory mechanism). In addition, Y292, Y315 and Y319 are thought to play an important scaffolding role, recruiting further SH2 domain containing proteins to the TCR signalling complex (Hsu *et al.*, 2009; Magnan *et al.*, 2001) (reviewed in (Au-Yeung *et al.*, 2009)). Direct targets of Zap70 kinase activity include the adaptor proteins linker of activated T-cells (LAT) (Zhang *et al.*, 1998), and SH2 domain containing leukocyte protein of 76kDa (Slp76) (Wardenburg *et al.*, 1996). These provide docking sites for a number of signalling molecules inducing further downstream signals (reviewed in (Samelson, 2002), and summarised in **Figure 1.3**).

A primary function of the TCR is to signal antigen recognition to the T-cells. TCR signalling induces an increase in the cytosolic concentration of calcium ions (referred to as calcium influx). TCR signals result in the activation of PLCγ, which acts to hydrolyse the membrane associated phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol

triphosphate (IP_3) (Yablonski *et al.*, 1998; Yablonski *et al.*, 2001). IP_3 induces opening of IP_3 gated calcium channels on the ER, resulting in store operated calcium entry (SOCE) (reviewed in (Vig & Kinet, 2009). The transient local increase in calcium is interpreted by calcium sensing domains on stromal interaction molecule (Stim) proteins. Through an unknown mechanism, the ER associated Stim proteins then interact with plasma membrane associated Orai calcium release-activated calcium modulator (Orai) proteins, resulting in multimerization and formation of a calcium channel at the plasma membrane (Luik *et al.*, 2008) (reviewed in (Hogan *et al.*, 2010)). What follows is a large influx of calcium ions, which act as second messenger through interactions with calcium binding domains, either directly or via calmodulin. Of particular importance in T-cells, this leads to the activation of the phosphatase calcineurin, which dephosphorylates and hence activates nuclear factor of activated T-cells (NFAT) proteins (reviewed in (Macian, 2005)).

TCR signalling also results in activation of mitogen activated protein kinase (MAPK) signalling cascades, either through the sequential recruitment/activation of growth receptor bound 2 (Grb2)/son of sevenless (sos)/Ras proto-oncogene (Ras) (reviewed in (Samelson, 2002)), or through DAG-mediated activation of ras guanyl nucleotide-exchanging protein (RasGRP)/Ras pathways (Dower *et al.*, 2000). In T-cells, the important MAPK pathway involved the sequential activation of Raf proto-oncogene serine/threonine-protein kinase (Raf), MAPK/Erk kinase (MEK) and Extracellular signal related kinase (Erk). Erk leads to the subsequent induction of the activator protein 1 (AP-1) transcription

factor (reviewed in (Mor & Philips, 2006)). Furthermore, TCR signalling activates the NFκB pathway through the DAG-mediated activation of Protein kinase C (PKC) (reviewed in (Baeuerle & Henkel, 1994)). NFAT, AP-I and NFκB are the classical targets of TCR signalling leading to T-cell activation.

However, the function of TCR signalling is not limited to T-cell activation, TCR signalling has been implicated in development, homeostasis, anergy and in cytoskeletal reorganisation following recruitment of non-catalytic region of tyrosine kinase adaptor protein-1 (Nck) and Vav oncogene (Vav) to LAT, and subsequent Vav activation (Costello *et al.*, 1999; Zeng *et al.*, 2003). As a further level of complexity, signals through the TCR induce negative feedback regulatory loops affecting TCR signal transducing complexes. This results in sensory adaptation and permits the resolution of true signals from background noise (reviewed in (Acuto *et al.*, 2008)). The context and nature of TCR signalling therefore results in divergent effector functions, but how a T-cell interprets these signals is not well-characterized.

1.4.2 The crossregulation of TCR and IL7R signalling

It has also been suggested that complex feedback mechanisms may influence TCR and IL7rα signalling and expression. IL7 signalling is regulated in T-cells by modulating the levels of IL7rα, whilst the γc is constantly expressed (reviewed in (Mazzucchelli & Durum, 2007)). It has been shown that IL7 signals downregulate IL7rα on naïve T-cells (Park *et al.*, 2004). Downregulation of the IL7rα in IL7-signalled T-cells may be 'altruistic', so that other T-cells receive less

competition from their recently signalled counterparts for this critical homeostatic factor (Munitic *et al.*, 2004). Furthermore, Park et al. have proposed a model termed coreceptor tuning, based on the finding that IL7 signalling stabilises CD8 coreceptor expression on lymph node T-cells (LNTs) *in vitro*. This model suggests that IL7 signals downregulate IL7rα, but upregulate CD8 coreceptor expression. High CD8 coreceptor expression was postulated to confer increased TCR signalling sensitivity. The authors further hypothesise that TCR signals block IL7R signalling and thus upregulate IL7rα, increasing IL7 signalling sensitivity. Such a cyclical model implies that T-cells temporally alternate between TCR and IL7 signalling during normal homeostasis (Park *et al.*, 2007). Although this model is intriguing, *in vivo* evidence relies largely on correlative data between IL7rα expression and the TCR signalling marker CD5 in TCR transgenic mice, which are commonly found to be lymphopenic (Azzam *et al.*, 2001; Ge *et al.*, 2004). The differences in endogenous IL7 levels in these mice may further complicate these conclusions. In addition, CD4 coreceptor expression was unaffected by IL7 signals, and the authors provided no explanation as to how CD4 homeostatic signalling is regulated (Park *et al.*, 2007). Therefore the physiological relevance of coreceptor tuning remains questionable.

In contrast to the findings of Park et al., Cho et al. recently found that TCR signalling augments IL7R signals, by inducing colocalisation of the IL7rα and γc in lipid rafts (Cho *et al.*, 2010). Also, Takada et al. found that in the absence of TCR:spMHC interactions, IL7rα levels were decreased on naïve T-cells

(Takada & Jameson, 2009). This contrasts findings in Lck-inducible transgenic mice, where the experimentally induced deletion of Lck has no affect on IL7rα expression levels (Seddon *et al.*, 2003). These seemingly paradoxical results are likely to result from differences in experimental systems used, and therefore the relationship between TCR and IL7 signalling is still unclear. Indeed, the molecular identities of putative factors involved in the cross-regulation of IL7rα and TCR signals have not yet been described.

1.4.3 Mechanisms of T-cell homeostasis

The signalling pathways activated by IL7R and TCR signalling are for the most part divergent, and the crossregulation between IL7R and TCR signalling pathways remains controversial. Nevertheless, both of these signalling pathways synergise to promote the homeostasis of T-cells. TCRα/β-deficient mice and IL7rα-deficient mice both exhibit severe blocks in T-cell lymphopoiesis, showing an essential role for both TCR and IL7R signals in this process (Peschon *et al.*, 1994; Philpott *et al.*, 1992) (see below, sections **1.5**-**1.7**). Peripheral homeostasis of naïve T-cells also depends on signals from cytokines and the TCR.

Mature T-cell numbers are maintained by protecting these cells from apoptosis (homeostatic survival). In addition, in a lymphopenic environment the cell turnover rate is modulated, so that there is a net gain of cells through proliferation (homeostatic proliferation) (Goldrath & Bevan, 1999; Kieper & Jameson, 1999; Sprent & Tough, 1994). Schluns et al. found a survival defect

in MHC-I-restricted TCR transgenic OT-I *Rag1*$^{-/-}$ *Il7r*$^{-/-}$ (IL7rα-deficient) T-cells relative to OT-I *Rag1*$^{-/-}$ controls, following transfer to an intact wild-type (WT) mouse. In addition, transfer of OT-I *Rag1*$^{-/-}$ cells to T-cell-deficient *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ *Il7*$^{-/-}$ (IL7-deficient) hosts showed impaired homeostatic proliferation in the absence of IL7 (Schluns *et al.*, 2000). WT CD4 and CD8 T-cells were also lost over an one-month period following transfer to IL7-deficient hosts (Tan *et al.*, 2001). Finally, a greater loss of naïve T-cells was observed following treatment of thymectomized mice with an anti-IL7R blocking-antibody, compared to untreated controls (Vivien *et al.*, 2001), and administration of IL7R-blocking antibody also impaired homeostatic proliferation of transferred T-cells in lymphopenic hosts (Seddon & Zamoyska, 2002a). Furthermore, IL7 signals play a role in CD4 and CD8 memory T-cell homeostasis (Kieper *et al.*, 2002; Seddon *et al.*, 2003). However, here IL15 plays a non-redundant role in the case of the CD8 memory cells (Kieper *et al.*, 2002) (reviewed in (Surh & Sprent, 2008)).

TCR signalling is also important for T-cell homeostasis, particularly in the case of naïve T-cells. Transferred H-2^{d}-restricted TCR transgenic T-cells were found to be short lived (with a half-life of ~3.5 weeks) when transferred to H-2^{b} hosts, but persisted in H-2^{d} controls, suggesting that TCR-restricted MHC interactions permit homeostatic survival (Kirberg *et al.*, 1997). Subsequent studies involving the conditional peripheral deletions of the TCRα-chain (Polic *et al.*, 2001), MHC-II (Witherden *et al.*, 2000), the CD4 coreceptor (Wang *et al.*, 2001) or Lck (Seddon *et al.*, 2000; Seddon & Zamoyska, 2002b) have confirmed a

requirement for TCR signalling in the survival and/or homeostasis of both CD4 and CD8 naïve T-cells. Interestingly, it has been hypothesised that the same ligands mediating homeostatic survival in the periphery are the same as those driving thymic development of T-cells (Ernst *et al.*, 1999). In contrast, in the absence of spMHC-II or TCR signalling CD4 memory T-cells survive, but show impaired homeostatic proliferation (Grandjean *et al.*, 2003; Seddon *et al.*, 2003). CD8 memory cells also persist in the absence of MHC-I (Murali-Krishna *et al.*, 1999). It is believed that memory cell homeostasis has a greater dependence on cytokine signals, predominantly mediated by IL7 and IL15 (reviewed in (Surh & Sprent, 2008)).

Therefore a wide variety of homeostatic mechanisms exist to maintain T-cells at a constant level, despite various inputs and outputs to different T-cell pools. Regulating the different T-cells pools by their requirement for both overlapping and unique factors enables both broad and subset-dependent mechanisms to operate. That such complex regulatory mechanisms exist highlights the importance of not just maintaining T-cell numbers, but also in maintaining clones exhibiting diverse polyclonal TCR repertoires. The elucidation of factors that govern this process are of clinical significance, with regards to improving immune function.

A critical requirement for T-cell homeostasis is the generation of T-cells through thymic development. The thymus produces a constant output of polyclonal T-cells, which can confer immunity against foreign antigen but are not self-reactive. This contrasts with homeostatic proliferation, which generates

monoclonal T-cell daughter cells. Thus it has been hypothesised that continued thymic development maintains a diverse repertoire of peripheral T-cells (reviewed in (Mackall *et al.*, 1997)). Furthermore, it is hypothesised that peripheral lymphoid cells reach a replicative senescence following a particular number of divisions due to shortening of the telomere in the absence of telomerase (referred to as the Hayflick limit) (Hayflick & Moorhead, 1961; Hayflick, 1965) (reviewed in (Effros, 1998)). Thymic generation may prevent replicative senescence by constantly generating new T-cells from stem cell precursors. Finally, the T-cell compartment is permanently reduced in neonatal thymectomized mice (reviewed in (Stutman, 1986)), whereas the peripheral T-cell pool is elevated following thymic engraftment (Berzins *et al.*, 1998). This suggests that the size of the naïve T-cell pool may be dictated by thymic export, although these studies do not rule out the contribution of thymically derived cytokines. In addition, both TCR and IL7 signals are essential for thymic development, showing that overlapping factors regulate both the generation and homeostasis of naïve T-cells. Therefore, identifying the mechanisms and processes underlying T-cell development has implications for understanding mature T-cell homeostasis.

1.5 Thymic development

The evolutionary appearance of the thymus correlates with the appearance of T-cells, in that it exists in all jawed vertebrates, but not lower species (reviewed in (Rodewald, 2008)). Thymic development of T-cells permits the generation of

a diverse polyclonal repertoire of T-cells. Given the highly effective T-cell effector response, it has been hypothesised that a dedicated organ may be necessary to reduce the potential for autoimmunity. This contrasts with other haematopoietic cell types, including B-cells, which predominantly develop in the BM (Boehm & Bleul, 2007).

The requirements of thymopoiesis include T-cell lineage commitment and generation of a diverse polyclonal T-cell repertoire that i) weakly recognises spMHC and ii) does not recognise spMHC with a strong enough avidity to lead to activation/autoimmunity. The thymus is also the site mediating the terminal differentiation of $\alpha\beta$-lineage T-cells to either the CD4 or CD8 lineage, as well as the differentiation of $\gamma\delta$ and NKT lineages. Finally effector function must be conferred to newly developed T-cells, so they leave the thymus fully capable of eliciting an immune response. These complex requirements are reflected in the intricate temporal sequence with which T-cell development progresses.

The temporal progression of $\alpha\beta$-T-cell development correlates with the changing surface phenotype of the maturing thymocyte populations. Cells are differentiated based on the expression of the CD4 and CD8 coreceptors. Initially thymocytes are $CD4^-CD8^-$ (referred to as double negative, DN). At the DN stage, thymocytes commit to the T-cell lineage, and rearrange their antigen receptor chains through somatic recombination of *Tcrb* followed by *Tcra* genetic loci. Thymocytes next enter a phenotypically $CD4^+CD8^+$ coreceptor double-positive (DP) stage. This is a developmental dead end for the majority of T-cells, as those cells receiving a negligible signal die by neglect. However,

those cells that recognise spMHC and receive relatively weak homeostatic TCR signals are positively selected to continue maturation. Following positive selection, T-cells subsequently commit to either the $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) lineages. Concomitantly with positive selection and CD4/CD8 SP lineage commitment, T-cells that recognise spMHC with a particularly strong avidity (signalling that they are self-reactive) are clonally deleted. Through these mechanisms, only T-cells that favourably recognise spMHC escape thymic development and are permitted to emigrate from the thymus to join the naïve T-cell pool.

1.6 Development of double negative thymocytes

The earliest requirement for T-cell development is the migration of thymus seeding progenitor (TSP) cells from the BM. However, the precise identity of these cells has remained elusive, and it has been suggested that multiple TSP populations may exist (reviewed in (Weerkamp *et al.*, 2006)). Following thymic migration these initially DN cells can be further subdivided into four stages based on the expression of CD44 and CD25 ($CD44^+CD25^-$; DN1, $CD44^+CD25^+$; DN2, $CD44^-CD25^+$; DN3 and $CD44^-CD25^-$ DN4) (Godfrey *et al.*, 1993). In the DN1 and DN2 stages, thymocytes progressively lose myeloid and B-cell potential and hence commit to the T-cell lineage. The upregulation of T-cell lineage-specific mRNA including Rag1, correlates with T-cell lineage commitment (reviewed in (Rothenberg *et al.*, 2008)). The extinction of alternative fate potential is finalised in the DN2 stage. Masuda et al. expressed

GFP from the Lck proximal promotor and found that GFP^+ DN2 cells represented T-cell lineage committed cells, whereas GFP^- DN2 cells retained DC differentiation potential (Masuda *et al.*, 2007). Following T-cell lineage commitment, DN2 cells somatically recombine either their *Tcrb* (encoding the TCR β-chain) or *Tcrg* and *Tcrd* (encoding the γδ-TCR) genes.

Those cells with in-frame γδ TCR rearrangements are subsequently signalled to commitment to the distinct γδ-T-cell lineage. γδ-T-cells are coreceptor negative, or express CD8αα homodimers, and recognise antigens in complex with classical (MHC-I/MHC-II) and non-classical MHC molecules, including T10 and T22 in mice (reviewed in (Hayday, 2000)). It has been suggested that γδ-T-cells recognise unique antigens and have a non-redundant role in both innate and adaptive immunity. For example, γδ-T-cell-deficient mice exhibit impaired mucosal IgA responses following oral administration of tetanus toxoid and cholera toxin (Fujihashi *et al.*, 1996), and show an increased susceptibility to *Candida albicans* infection (Jones-Carson *et al.*, 1995).

Nevertheless, γδ-T-cells represent a minority population in the thymus. The majority of DN2 thymocytes will rearrange TCRβ and surface expression occurs along with the invariant pre-TCRα chain (referred to as the pre-TCR hereon) at the DN3 stage (Groettrup *et al.*, 1993). Commitment to the αβ-T-cell lineage over the γδ-lineage is finalized by pre-TCR signalling (Fehling *et al.*, 1995). Two recent papers suggest that αβ versus γδ-lineage commitment is likely to depend on the relative TCR signalling strengths conferred by the pre-TCR versus the

γδ-TCR. Hayes et al. found γδ-TCR transgenic mice exhibited αβ-lineage committed DP cells due to low transgenic γδ-TCR expression. CD5 is a negative regulator of TCR signalling and therefore crossing the mice onto a CD5-deficient background increased signalling and was also found to reduce γδ to αβ-lineage misdirection (Hayes *et al.*, 2005). In addition Haks et al. found that attenuation of TCR signalling resulting from Lck-deficiency redirected γδ-TCR transgenic mice to the αβ-lineage (Haks *et al.*, 2005). These data suggest that γδ-lineage commitment is mediated by relatively strong TCR signalling, whereas αβ-commitment is mediated by relatively weaker pre-TCR signalling. If such a model proves to be correct, it would show that T-cells make a developmental fate decision based on the interpretation TCR signal strength.

In addition to cementing αβ-T-cell lineage fate, pre-TCR signalling induces the rearrangement and expression of the TCRα chain at the DN3 stage of development (Koyasu *et al.*, 1997). What follows is a proliferative burst and a downregulation of CD25 as cells phenotypically transit to the DN4 subpopulation (Penit *et al.*, 1988; Vasseur *et al.*, 2001). CD4 and CD8 coreceptor expression is subsequently induced, and these coreceptors are expressed with asymmetric kinetics, so cells transit through a coreceptor skewed immature single positive (ISP) stage on the way to becoming $CD4^+CD8^+$ coreceptor double-positive (DP) cells (Paterson & Williams, 1987). Therefore the major driving force behind DN to DP maturation in αβ-T-cells is the successful rearrangement of the TCRβ-locus and signalling mediated by the pre-TCR, known as the β-selection checkpoint.

In addition to TCR signalling, IL7 signals also play a role in DN T-cell development, where they augment pre-TCR signals to promote survival. There is severe reduction in DN αβ-lineage T-cells in IL7 or IL7rα-deficient mice (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995), however transgenic expression of the pro-survival molecule Bcl2 rescues αβ, but not γδ-T-cell numbers (Akashi *et al.*, 1997). Therefore, IL7 signals are hypothesised to play a role in γδ-T-cell development beyond promoting survival. These data also suggest that in addition to limiting the naïve T-cell pool numbers, IL7 also limits the production of T-cell precursors. Fitting with this suggestion, early thymic precursors are increased in IL7 transgenic mice (Samaridis *et al.*, 1991).

1.7 Double positive thymocyte development

The second TCR-signalling-dependent checkpoint in the development of T-cells occurs at the DP stage. The DP population is a developmental dead-end for the majority of T-cells, which do not express in-frame TCRα rearrangements that can successfully pair with the TCRβ-chain. These cells cannot receive a spMHC-mediated signal, resulting in their death by neglect. In contrast those cells receiving a particularly strong TCR:spMHC signal are clonally deleted (referred to as negative selection), a mechanism to prevent autoreactive cells from gaining effector function (Kappler *et al.*, 1987). Only those cells receiving a relatively weak spMHC signal, likened to a homeostatic TCR signal are positively selected to develop to maturity (reviewed in (Starr *et al.*, 2003)).

1.7.1 Death by neglect

In the absence of MHC molecules, T-cells are arrested at the DP stage (Grusby *et al.*, 1993), showing that TCR:spMHC signals facillitate the positive selection of DP cells. In addition, DNA labelled DP cells were found to have a 3.5 day lifespan (Huesmann *et al.*, 1991). Apoptosis of DP cells has been visualized *in situ* in the thymic cortex of MHC-deficient mice (Surh & Sprent, 1994), showing that DP cells will die in the absence of MHC. Thus it follows that T-cells that cannot contact spMHC will die by neglect at the DP stage. Further evidence for this comes from the TCRα-deficient mice, that show a DP arrested phenotype, but no accumulation of DP cells (Mombaerts *et al.*, 1992a). DP cells are particularly sensitive to apoptosis, as they do not express IL7rα (Sudo *et al.*, 1993), express high levels of suppressor of cytokine signalling 1 (SOCS1) (Chong *et al.*, 2003) and are refractive to IL7/γc-cytokine survival signals (Van De Wiele *et al.*, 2004). Furthermore, non-positively selecting DP cells express low levels of the prosurvival molecule Bcl2 (von Freeden-Jeffry *et al.*, 1997). Death by neglect ensures that T-cell clones incapable of self-recognition are rapidly deleted. This is important because T-cells detect foreign antigen in the context of MHC.

1.7.2 Negative selection

A number of experimental models suggest that strong recognition of spMHC results in the clonal deletion of DP cells. Strong signalling induced by anti-CD3 treatment (Smith *et al.*, 1989), or by exogenous administration of agonist TCR ligand in TCR transgenic mice induces the rapid and efficient deletion of DPs

(Sebzda *et al.*, 1994). Furthermore, it has been found that negatively selecting ligands have a higher affinity for the TCR compared to positively selecting ligands (Alam *et al.*, 1996). Recent evidence suggests that clonal deletion to self-antigen can occur in the thymic cortex, and is likely to be efficiently mediated by a $CD11c^+$ DC cell subset (McCaughtry *et al.*, 2008), although the unique self-antigens expressed in the thymic medulla are also essential for negative selection (see below, section **1.10.2**). It is thought that the strong signals in the DP stage induce differential downstream signalling pathways than those mediated by positive selection signals, allowing a cell to distinguish between positive and negative selection (reviewed in (Starr *et al.*, 2003)). Thus only those cells with a weak avidity for spMHC undergo positive selection and progress beyond the DP stage of development.

1.8 CD4/CD8 single positive lineage specification

1.8.1 Positive selection

Only cells with a weak affinity for spMHC undergo positive selection at the DP stage. This is accompanied by phenotypic changes to the selecting cell, including upregulation of the activation markers CD5 and CD69 (Azzam *et al.*, 1998; Lucas & Germain, 1996; Swat *et al.*, 1993). Positively selecting cells also undergo a developmental lineage choice, committing to either the CD4 or CD8 lineage. Lineage commitment is directed by the nature of the positive selection signal. Those cells recognising spMHC-II subsequently commit to the CD4 SP lineage, whereas those cells recognising spMHC-I commit to the CD8 SP

lineage. Positive selection has been viewed solely as a developmental event. It is unknown whether different clonal spMHC avidities have an impact on this process, or whether positive selection impacts the future homeostatic potential of a selecting T-cell clone. Furthermore, the question of how T-cells interpret MHC-II versus MHC-I-mediated positive selection signals has been the subject of much contention. A number of different models have been proposed to explain this process.

1.8.1 Stochastic selection model

The stochastic model of T-cell development proposes that CD4 or CD8 lineage commitment occurs at random. This is followed by a coreceptor-mediated "rescue" stage, which ensures that only those SP cells with a matching TCR/coreceptor:MHC interaction survive. Support for this model was gained following identification of $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ populations in both MHC-II-deficient and MHC-I-deficient mice, suggested to represent developmentally intermediate cells of a mismatched lineage (Chan *et al.*, 1993). Evidence for the stochastic model also came from "coreceptor rescue" experiments, whereby transgenic expression of coreceptors gave rise to SP cells of a mismatched TCR:MHC specificity, albeit with a low efficiency (Davis *et al.*, 1993; Itano *et al.*, 1994).

The stochastic model therefore predicts that CD4/CD8 lineage commitment would be a highly error prone process, as 50% of positively selected cells would be expected to die following extinction of matched coreceptor expression. The

finding that positive selection efficiency could approach 90% in TCR transgenic mice therefore opposes the stochastic model (Itano & Robey, 2000). Furthermore, in the helper-deficient (HD) mice, which have a defect in CD4 lineage commitment, MHC-II-restricted cells were redirected to the CD8 lineage and exported to the periphery (Keefe *et al.*, 1999). This paper suggested that cells with mismatched coreceptors were not short-lived in the absence of coreceptor:MHC interaction. Furthermore targeting of CD4 coreceptor expression specifically to CD8 lineage cells did not result in increased CD8 SP development, further arguing against coreceptor-mediated rescue (Adoro *et al.*, 2008). These results are paradoxical compared to initial coreceptor-rescue experiments, and it has been argued that the non-physiological nature of the transgene expression in the initial studies may result in TCR signal misinterpretation leading to alternative lineage choice (reviewed in (Singer *et al.*, 2008)). When taken together, evidence suggests that lineage commitment is more likely to be instructed by differences in MHC-II or MHC-I-mediated positive selection signals.

1.8.2 Instructive models of lineage specification

Instructive models of lineage commitment suggest that differential signalling mediated by MHC-II versus MHC-I instructs the CD4/CD8 lineage commitment decision. Initially it was proposed that differential recognition of MHC-II or MHC-I resulted activated divergent TCR signalling cascades (Robey *et al.*, 1991; Seong *et al.*, 1992). The lack of evidence for differential signalling led to the refinement of this model, to suggest that quantitative differences in TCR

signalling strength underlie the CD4 versus CD8 lineage decision (Matechak *et al.*, 1996).

1.8.2.1 Quantitative instructive model

The quantitative instructive model was first proposed to reconcile the finding that MHC-II restricted T-cells develop to the CD8 lineage in the absence of the CD4 coreceptor. It was suggested that loss of CD4 lowered the TCR:MHC avidity, resulting in a weaker signal (Matechak *et al.*, 1996). Thus CD4 lineage development is associated with stronger positive selection signalling, whereas CD8 SP development is associated with weaker signalling. Underlying this model is the finding that the cytoplasmic tail of CD4 has a stronger binding affinity for Lck than does CD8 (Wiest *et al.*, 1993). Culture of thymocytes in neonatal thymic organ cultures (NTOCs) along with weak agonist ligands resulted in CD4 development (Bommhardt *et al.*, 1997), whereas culture with antagonist ligands resulted in CD8 development (Basson *et al.*, 1998). Both constitutively active and overexpressed Lck resulted in preferential development of CD4 SP cells (Hernandez-Hoyos *et al.*, 2000; Legname *et al.*, 2000), whereas expression of a dominant-negative form of Lck favoured CD8 SP development (Hernandez-Hoyos *et al.*, 2000). Furthermore, Lck-deficiency results in an incomplete block in both β-selection and positive selection. Lck-deficient mice show an increased frequency of CD8 SPs relative to CD4 SPs in both the thymus and periphery (Molina *et al.*, 1992).

There is however conflicting evidence that cannot be explained by a simple quantitative instructive model. For example mutation of ITAMs on CD3ζ-chains

was found to reduce TCR signalling capacity without skewing the CD4 versus CD8 lineage frequency (Holst *et al.*, 2008; Love *et al.*, 2000). Also, generation of mice expressing chimeric CD8α molecules with CD4 cytoplasmic tails resulted in a greater recruitment of Lck to the coreceptor tails, but no CD4 lineage redirection in MHC-I signalled thymocytes (Bosselut *et al.*, 2001; Erman *et al.*, 2006). In addition, despite differences in proximal signalling strength associated with CD4 versus CD8 SP development, how a selecting cell integrates and interprets these signals remains unclear. Given the wide range of potential spMHC avidities, it is unclear how a T-cell could distinguish a weak avidity MHC-II signal from a strong avidity MHC-I signal. A diagram summarizing the quantitative instructive model is shown in **Figure 1.4 A**.

1.8.2.2 Kinetic signalling model

The kinetic signalling model was proposed as an alternative to the quantitative instructive model of CD4/CD8 lineage commitment. Critical experiments leading to the formulation of this model came from Yasutomo et al., who showed that in an *in vitro* culture system, the duration of contact with self-antigen presenting DCs correlated with subsequent DP cell lineage choice. Whereas longer contact promoted CD4 lineage development, shorter contact promoted CD8 lineage development (Yasutomo *et al.*, 2000). It was previously shown that phenotypically intermediate populations of positively selecting cells could be identified based on modulation of coreceptor expression. These cells have begun the process of positive selection, but are not yet committed to either the CD4 or CD8 lineage, and are phenotypically $CD4^{hi}CD8^{lo}$ (Lucas & Germain,

1996; Lundberg *et al.*, 1995). Brugnera et al. integrated these findings, suggesting that cells recognising MHC-II, which depended on the CD4 coreceptor received continuous signals at the $CD4^{hi}CD8^{lo}$ stage. In contrast, the termination of the CD8 coreceptor expression in $CD4^{hi}CD8^{lo}$ cells was hypothesised to interrupt and shorten TCR signalling, resulting in the CD8 SP fate decision (Brugnera *et al.*, 2000). This model represented a step forward from 'classical' lineage commitment models, as for the first time it took into account the kinetic distinction between initiation of positive selection and subsequent lineage commitment (reviewed in (Singer *et al.*, 2008)). *In vivo* evidence for the kinetic signalling model came the $CD4^{E8III}$ mouse, where the CD4 coreceptor is under control of CD8 expression elements. These mice showed premature termination of the CD4 coreceptor following positive selection, and developed MHC-II restricted CD8 SP lineage cells (Sarafova *et al.*, 2005).

It has additionally been proposed that IL7 provides the differentiation signal needed to instruct CD8 SP development from $CD4^{hi}CD8^{lo}$ cells. Evidence for this first came from a two-step culture system *in vitro*. Following Phorbol 12-myristate 13-acetate and ionomycin treatment, DP cells cultured with IL7 in the secondary culture apparently developed into CD8 SP cells with a greater efficiency then cells cultured with medium alone (Yu *et al.*, 2003). It has been additionally suggested that IL7 induces Runt-related transcription factor (Runx)3 gene expression, a factor critical for CD4 silencing and CD8 SP development (Park *et al.*, 2010) (see below, section **1.9.1**).

There are however discrepancies between the kinetic signalling model and some experimental data. When Zap70 was expressed under control of adenosine deaminase (ADA) expression elements, expression was restricted to early DP cells and was terminated upon initiation of positive selection (Liu *et al.*, 2003). It was found that premature termination of Zap70 expression alone did not induce CD8 SP lineage fate in the absence of transgenic Bcl2 expression, leading researchers to argue for a TCR-mediated "proofreading" step in CD8 development (Liu *et al.*, 2003; Liu & Bosselut, 2004). In addition, there is no obvious change in the CD4/CD8 ratio in the IL7 or IL7rα-deficient mice compared to controls (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995), and CD8 SP development is still permitted in γc-deficient mice (Di Santo *et al.*, 1999), suggesting that any instructive role played by IL7 may be redundant. Furthermore, it has been argued that the kinetic signalling model and quantitative instructive models are not mutually exclusive (reviewed in (He *et al.*, 2010)). Premature termination of TCR signalling and quantitative differences in TCR signal strength may represent redundant mechanisms, enabling a greater degree of accuracy during the CD4/CD8 lineage decision. Finally, whilst this model classifies positive selection as a distinct event from lineage commitment, the lack of phenotypic markers other than CD4 and CD8 precludes the reliable identification and analysis of developmentally intermediate subsets of cells. A diagram summarizing the kinetic signalling model is shown in **Figure 1.4 B**.

1.9 Single positive lineage commitment

In addition to 'top-down' approaches that examined the proximal signalling events regulating the CD4/CD8 lineage decision, there has been an increasing focus on the downstream factors responsible for cementing CD4 and CD8 lineage commitment. These so-called 'bottom-up' approaches have identified numerous factors critical for the development of one or other αβ-T-cell lineage. For example the transcription factors GATA-binding protein 3 (Gata3) and T-helper-inducing *POZ*/Krueppel-like factor (ThPOK) are essential for CD4 lineage development, whereas Runx3 is required for CD8 development.

1.9.1 CD8 lineage commitment

Runx proteins were first implicated in the CD4/CD8 lineage decision by their essential role in silencing of *Cd4* gene transcription in CD8 lineage cells (Taniuchi *et al.*, 2002). Deletion of Runx3 reduced CD8 SP generation (Woolf *et al.*, 2003), and the subsequent generation of Runx3-deficient mice with a CD4-Cre-mediated DP deletion of the redundant factor Runx1 revealed an essential role for Runx factors in CD8 lineage development (Egawa *et al.*, 2007). Overexpression of Runx3 increased the proportion of mature CD8 SP thymocytes, although it did not entirely ablate CD4 lineage development (Kohu *et al.*, 2005). In addition, enforced Runx3 expression did not induced CD8 lineage differentiation in unsignalled DP thymocytes (Park *et al.*, 2010). This suggests the role of other non-redundant factors involved in the selection of CD8 lineage cells. Interestingly, Runx proteins have been implicated in playing

an antagonistic role in the regulation of the CD4 lineage-commitment factor ThPOK (reviewed in (Collins *et al.*, 2009) and see below).

In addition, a recent study Jones and Zhuang generated mice that conditionally deleted the E proteins E2A and HEB in DP thymocytes. In these mice DP cells spontaneously developed into CD8 lineage cells that emigrated to LNs, even in the absence of MHC (Jones & Zhuang, 2007). However, the relationship between E proteins, TCR signaling and other transcriptional networks implicated in the CD4/CD8 lineage decision remains to be clarified.

Other than a non-redundant role for Runx proteins and potentially the negative regulation of E proteins, not much is known of the factors and mechanisms required for CD8 development. When thymus high-mobility box protein (Tox) was overexpressed, it was found to block CD4 SP development, leading to the conclusion that this factor may be involved in determining CD8 lineage fate (Aliahmad *et al.*, 2004). However, CD4 SPs were paradoxically absent in Tox-deficient mice, whereas CD8 SP development was intact (Aliahmad & Kaye, 2008). The role of Tox is therefore unclear, but it certainly does not appear to play a non-redundant role in CD8 SP fate specification. Similarly, it was thought that notch signalling played a role in instructing lineage commitment, based on gain of function studies. However, loss-of-function studies revealed no redirection of MHC-II-restricted thymocytes to the CD8 lineage, suggesting that rather than lineage commitment, notch may be promoting CD8 lineage survival (reviewed in (Laky & Fowlkes, 2008)). Indeed

the studies with notch reveal the difficulty in discriminating lineage commitment factors, from factors differently involved in CD4 and CD8 homeostasis.

1.9.2 CD4 lineage commitment

The transcription factor Gata3 is dispensable for positive selection, but necessary for CD4 SP lineage commitment. Transgenic overexpression of Gata3 blocked CD8 SP lineage development in fetal thymic organ cultures (FTOC). In contrast, inhibiting Gata3 expression through transfection of small inhibitory RNA (siRNA) or Gata3 function through overexpression of a Gata3 antagonist blocked CD4 SP lineage commitment (Hernandez-Hoyos *et al.*, 2003). It was subsequently confirmed that Gata3 is essential for CD4 SP but dispensable for CD8 lineage development, in mice where Gata3 was conditionally deleted at the DP stage using a CD4-cre/Loxp-mediated system (Pai *et al.*, 2003). Gata3 was shown to be downstream of TCR signalling in DP cells, perhaps induced or augmented by the cMyb transcription factor (Maurice *et al.*, 2007). Thus a link may exist between TCR signalling strength/length and induction of a factor essential for CD4 SP lineage development.

Examination of a spontaneous HD mouse mutant that lacked CD4 but not CD8 lineage T-cells revealed another essential transcription factor for CD4 lineage development (Dave *et al.*, 1998a; Keefe *et al.*, 1999). Genetic mapping revealed a loss-of-function point-mutation in a DNA binding domain of the factor ThPOK, and ectopic ThPOK expression led MHC-I-restricted thymocytes to adopt a CD8 lineage fate. Therefore it was suggested that ThPOK is a

'master-regulator' of CD4 lineage commitment (He *et al.*, 2005). Although the mode of action of ThPOK on the lineage commitment decision is not well characterized, it has been suggested to oppose Runx-mediated repression of a CD4 silencer region, thus promoting maintenance of CD4 coreceptor expression (Wildt *et al.*, 2007). ThPOK expression depends on the function of cis-acting enhancer and repressor regions (reviewed in (He *et al.*, 2010)). Deletion of two conserved Runx binding sites in the *Thpok* repressor region was associated with Thpok-reporter gene de-repression in the CD8 lineage (Setoguchi *et al.*, 2008), although other researchers report no effect from deleting this region (He *et al.*, 2008). In contrast it has been reported that Gata3 is downstream of TCR signalling and upstream of ThPOK induction during CD4 lineage commitment (Maurice *et al.*, 2007; Muroi *et al.*, 2008; Wang *et al.*, 2008). Two conserved Gata3 binding sites exist in the *Thpok* promotor sequence, although their function remains to be tested in knockout studies. Thus Gata3 has been suggested to act as a lineage-specifying factor, whereas ThPOK is suggested to play a role in lineage commitment (Wang *et al.*, 2008). Future work will undoubtedly reveal additional factors regulated by ThPOK, however ThPOK appears to be prominently positioned in a transcriptional network involving many of the key players in the CD4/CD8 lineage decision.

The experimental evidence to date has therefore identified a number of key transcription factors involved in the CD4/CD8 lineage commitment decision. Future work must focus on addressing i) What are the upstream regulators of such factors, and how ultimately they are regulated by TCR signalling, in

addition to ii) What are the downstream target genes for these lineage commitment factors. The subdivision of the CD4/CD8 lineage commitment decision into distinct positive selection, lineage specification and finally lineage commitment stages has broadened the understanding of the transcriptional networks involved. Examination of the various lineage commitment factors with a finer temporal resolution may enable further insights to their functions.

1.10 Single positive maturation

Following lineage specification and commitment, thymocytes are phenotypically CD4 or CD8 SP, but still require further maturation. Whereas DN and DP T-cell development occurs in the thymic cortex, lineage committed thymocytes rapidly migrate to the medulla (Witt *et al.*, 2005), where they are exposed to an environment that uniquely expresses tissue-restricted antigens (TRAs) (reviewed in (Anderson *et al.*, 2007)). The most immature SP thymocytes express high levels of Heat-stable antigen (HSA, CD24), and are subject to clonal deletion following strong TCR stimulation (Kishimoto & Sprent, 1997). Cells that strongly recognise self-antigens expressed in either the thymic cortex or medulla are rapidly deleted. Alternatively, the remaining cells that do not exhibit strong reactivity to self-antigen gain the potential to elicit an immune response, and subsequently emigrate to peripheral lymphoid tissues.

1.10.1 Migration from the thymic cortex to medulla

DN and DP thymocytes reside in the thymic cortex, whereas SP thymocytes relocate to the thymic medulla (reviewed in (van Ewijk, 1991; Yin *et al.*, 2006)).

Evidence suggests that the relocation of positively selected SP thymocytes to the thymic medulla involves their long-range and directed migration towards chemotactic factors produced in the medulla (Witt *et al.*, 2005). This occurs because positive selection induces changes in chemokine sensitivity. For example, treatment of cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin at concentrations known to mimic the positive selection signal induces the upregulation of CCR7 (Adachi *et al.*, 2001), which is also upregulated *in vivo* following positive selection (Misslitz *et al.*, 2004). CCR7 is the receptor for C-C motif chemokine ligand (CCL)19 and CCL21 (Yoshida *et al.*, 1997), which are highly expressed by the thymic medulla (Misslitz *et al.*, 2004; Ueno *et al.*, 2002). The upregulation of CCR7 correlates with an increase in sensitivity to CCL19 and CCL21 (Campbell *et al.*, 1999; Kim *et al.*, 1998a). Furthermore, CCR7-deficient mice or mice with a spontaneously arising deletion of both CCL19/CCL21 genes, first identified as exhibiting a paucity of lymph node T-cells (referred to as *plt/plt* mice), exhibit an accumulation of SP cells in the thymic cortex (Misslitz *et al.*, 2004; Nakano *et al.*, 1998; Ueno *et al.*, 2004). Hence CCR7:CCL19/CCL21 interactions are essential to mediate homing of SP thymocytes to the medulla.

The question arises as to what the role of cortical to medullary migration is in thymocyte development. Crosstalk between SP thymocytes and medullary thymic epithelial cells (mTECs) is required for mTEC terminal differentiation (reviewed in (Anderson & Jenkinson, 2001)) and in keeping with this phenomenon; CCR7-deficient and *plt/plt* mice additionally exhibit dysregulated

thymic medullary architecture (Misslitz *et al.*, 2004). Furthermore, the medulla represents a unique thymic microenvironment where TRAs are ectopically expressed, facilitating the deletion of autoreactive thymocytes before they reach maturity (see below, section **1.10.2**). Indeed SP cells developing in CCR7-deficient mice showed increased self-reactivity, causing potent dacryoadenitis and sialadenitis (Kurobe *et al.*, 2006). This finding suggests that the directed migration of positively selected thymocytes to the thymic medulla is a requirement for central tolerance. Thus, following positive selection, the thymus can influence the T-cell repertoire before maturation is completed.

1.10.2 Negative selection in the thymic medulla

DP and newly generated SP cells are subject to deletion following stimulation with a strong TCR signal (Kishimoto & Sprent, 1997), suggesting that negative selection can occur in both the thymic cortex and the medulla. Indeed, of the TCR transgenic mice that have been used to model clonal deletion, approximately half were found to delete in the cortex and the other half in the medulla (reviewed in (Hogquist *et al.*, 2005)). However, the frequency of apoptotic cells found in the thymic cortex was identical in MHC-deficient and MHC-sufficient polyclonal mice, suggesting that medullary deletion has a greater physiological relevance with regards to negative selection (Surh & Sprent, 1994). That cells with defects in medullary migration exhibit autoimmunity provides further evidence for the essential role of the thymic medullary environment in establishing central tolerance (Kurobe *et al.*, 2006).

Autoimmune polyendocrinopathy-candidiases-ectodermal dystrophy (APECED) is a rare genetic disease in humans characterised by chronic mucocutaneous candidiases, hypoparathyroidism and adrenal insufficiency, resulting in a failure of central tolerance (reviewed in (Mathis & Benoist, 2009)). The genetic mutations responsible for this condition were identified in a gene subsequently named autoimmune regulator (AIRE) (Aaltonen *et al.*, 1997). Knocking out *Aire* in mice similarly resulted in autoimmunity (Anderson *et al.*, 2002). Aire is hypothesised to function as a transcription factor, mediating thymic medullary expression of TRAs (reviewed in (Mathis & Benoist, 2009). Hence, in the thymic medulla, those T-cells strongly recognising TRAs will be clonally deleted before reaching maturity (reviewed in (Hogquist *et al.*, 2005)). In contrast, AIRE-deficiency leads to a break in central tolerance and permits the maturation of autoreactive T-cell clones.

1.10.3 Further differentiation of CD4 T-cells

The thymic medulla is also likely to be the site of further differentiation of the CD4 lineage. The majority of CD4 SP thymocytes will remain committed to the $\alpha\beta$-T-cell lineage, however this population also represents the likely branch point for thymically derived Tregs cells and NKTs.

Tregs represent a distinct lineage of $\alpha\beta$-T-cells, characterised by their expression of Foxp3, in addition to other activation markers (reviewed in (Sakaguchi, 2004). It is thought that Treg development occurs from CD4 SP precursors that have a relatively strong avidity for spMHC, above the threshold

for positive selection, but just below the negative selection threshold. Currently, a two-step model of Treg development is favoured, suggesting that strong TCR signalling upregulates CD25, facilitating enhanced IL2 sensitivity (Lio & Hsieh, 2008). This permits subsequent signalling from IL2, required to cement Treg lineage fate (reviewed in (Josefowicz & Rudensky, 2009).

NKT cells also represent a conserved CD4 SP-derived T-cell sub-lineage. These cells express an invariant TCRα-chain and are restricted to CD1d, a molecule related to MHC-I. These are thought to represent T-lineage cells, however they additionally express the NK-cell marker NK1.1 (reviewed in (Kronenberg, 2005)). Although the physiological relevance of NKT cells is not well characterised, their development along with Tregs from CD4 SP cells further highlights the role of the thymus in shaping the peripheral T-cell repertoire, by facilitating signals that divert SP cells to particular lineages (reviewed in (Godfrey & Berzins, 2007)).

1.10.4 Thymic egress

A final hurdle for developing T-cells is their emigration from the thymus. It has been estimated that $5x10^7$ T-cells emigrate from the thymus per month in young adult mice (Scollay *et al.*, 1980). However, these cells must compete for integration into peripheral niches, so the exact contribution of recent thymic emigrants (RTEs) to the naïve pool in a full host remains unknown. Peripheral T-cell numbers are maintained in thymectomized adult mice (Tough & Sprent, 1994), although in the absence of thymic development there is a relative

increase in the $CD44^{hi}$ memory-like phenotype cells, and a corresponding decrease in naïve cells (Tanchot & Rocha, 1995). In addition, the loss of thymic output may also be associated with a decrease in naïve T-cell repertoire diversity, which may increase disease susceptibility (reviewed in (Mackall *et al.*, 1997)).

Thymic emigration requires upregulation of sphingosine-1-phosphate receptor-1 (S1P1) on T-cells and occurs in a sphingosine-1-phosphate (S1P) dependent manner (reviewed in (Weinreich & Hogquist, 2008)). Although S1P1-deficient mice die of vascular defects *in utero* (Pilorget *et al.*, 2007), chimeric mice reconstituted with S1P1-deficient fetal liver cells showed relatively normal thymocyte development, but no thymic egress (Matloubian *et al.*, 2004). Zachariah and Cyster have recently shown that neural-crest derived pericytes, which ensheath blood vessels at the cortico-medullary junction provide an essential source of S1P to mediate egress (Zachariah & Cyster, 2010). The mechanisms underlying the kinetics of S1P1 expression on newly generated SP thymocytes are unknown (reviewed in (Weinreich & Hogquist, 2008)), although this process is thought to occur over several days following positive selection (Le Campion *et al.*, 2000; McCaughtry *et al.*, 2007). Nonetheless, the regulation of thymic egress has relevance to the question of how long a SP cell is retained in the thymic medulla and is thus amenable to negative selection.

1.11 Thesis aims

That T-cells require a specialised organ to facillitate their development highlights the complexity of this selective process. The thymic microenvironment facilitates the random rearrangement of TCRs, which are then screened to ensure sufficient but not overt levels of self-reactivity. Thymocytes are subsequently directed to either the CD4 or CD8 lineage, based on the MHC-restriction of their TCR.

How a CD4 or CD8 SP fate decision is made by a DP thymocyte has been the subject of extensive research. It is recognised that positive selection and CD4/CD8 lineage commitment occurs over several days. However, a lack of phenotypic markers that can reliably identify developmentally intermediate populations of selecting thymocytes has proven to be a major obstacle in studying this process. There is a consensus that differences in TCR signalling likely conferred by the CD4 or CD8 coreceptor instruct a DP cell to commit to either SP lineage. However, the precise nature of these disparate signals remains intensely debated, with recent models suggesting that quantity or duration of TCR signalling plays a role. Furthermore, such models do not address whether there is a temporal component to the integration of lineage commitment signals. For example, it is unknown whether CD4 and CD8 SP development occurs with identical kinetics following positive selection.

The nature of the positive selection signal is also likely to vary within the same lineage, as unique T-cell clones are likely to have a different avidity for spMHC

and may be subject to different levels of clonal competition. It is unknown whether the quality of the positive selection impacts on the homeostatic survival potential of a selected T-cell, or whether all positively selected T-cell clones have an equal chance of peripheral survival. Researching the mechanisms of thymic development is essential to understand how a diverse peripheral T-cell repertoire is maintained.

Thus we aimed to investigate three broad aspects of thymic development:

i) The kinetics of the CD4 versus CD8 lineage decision, and the phenotypic changes associated with this event.

ii) The interpretation of the positive selection TCR signal by selecting thymocytes.

iii) The impact of the positive selection signalling strength on the future homeostatic survival potential of mature T-cells.

Figure 1.1 IL7R signaling pathways

A schematic representation of signalling pathways activated downstream of the IL7-receptor (IL7R). IL7 binding induces phosphorylation of Janus kinase (JAK)1, JAK3 and subsequently IL7rα-chain. This resultant signalling cascades lead first to signal transducer and activator of transcription (STAT)5 recruitment to phosphorylated immunotyrosine activation motif (ITAM)s on the IL7rα chain. Recruited STAT5 is subsequently phosphorylated by JAK1/JAK3, facilitating its dimerization and subsequent nuclear translocation. Secondly, IL7 signalling induces phosphatidylinositol-3-kinase (PI3K) recruitment to the IL7rα chain. This recruitment brings PI3K in close proximity with the plasma membrane and permits the phosphorylation of phosphatidylinositol 4,5,-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5,-triphosphate (PIP_3). Akt and its activating kinase 3-phosphoinosite-dependent protein kinase-1 (PDK1) both possess plekstrin homology (PH) domains, that facillitate lipid binding and thus co-localization with PIP_3. The co-localization of PDK1 and Akt results in Akt activation, and induction of downstream signalling cascades leading to a pro-survival, cell growth and cell proliferation response. Transcription factors activated by IL7R signalling are shown in shaded red.

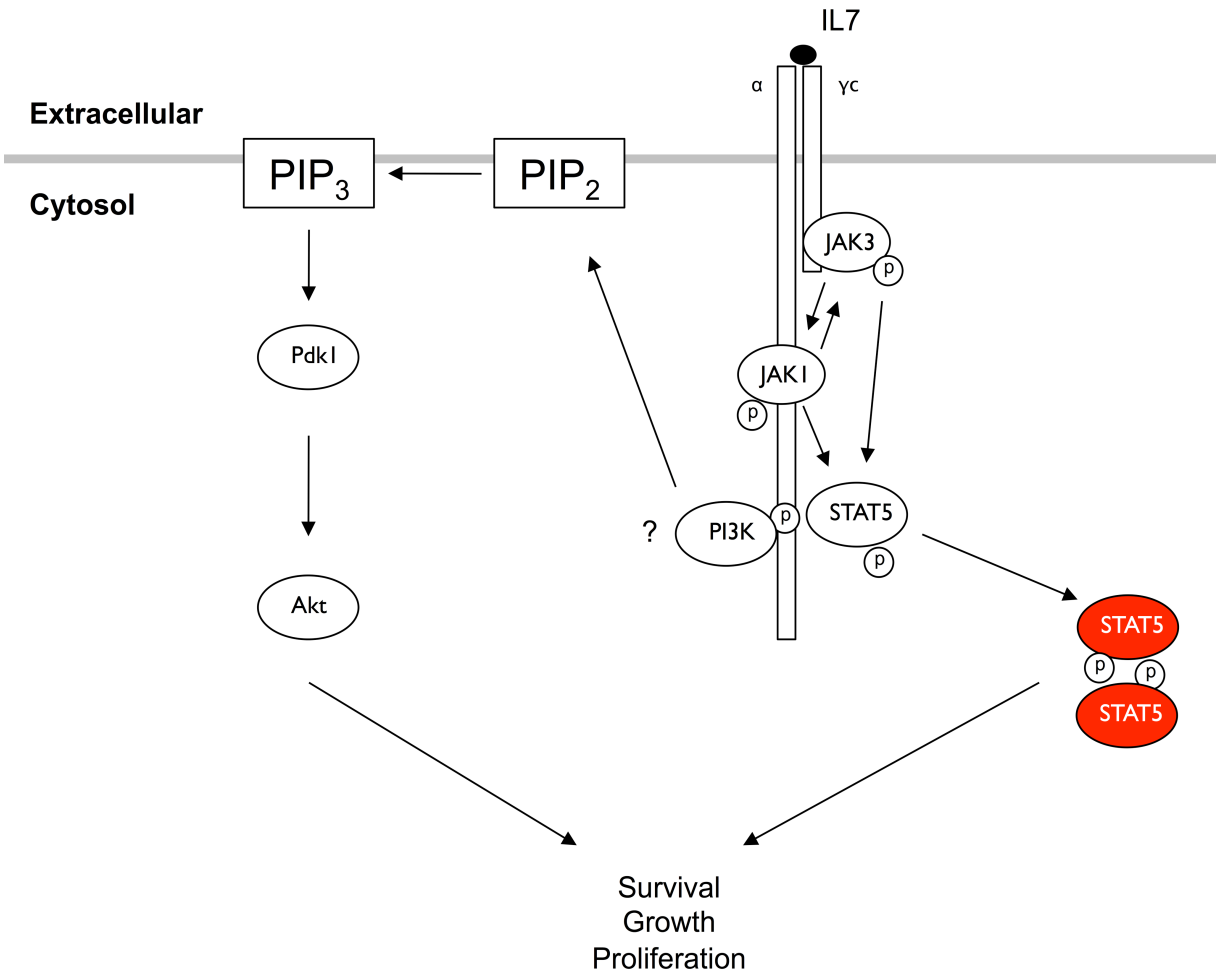

IL7
α
γc
Extracellular
Cytosol
PIP3
PIP2
JAK3
P
Pdk1
JAK1
P
?
PI3K
P
STAT5
P
Akt
STAT5
P
P
STAT5
Survival
Growth
Proliferation

Figure 1.2 The structure of Zap70

A schematic diagram showing Zap70 domains, and important tyrosine residues involved in signal transduction. Zap70 consists of two N-terminal SH2 domains (shaded salmon) and a C-terminal kinase domain (shaded blue), separated by a flexible linker domain (interdomain B).

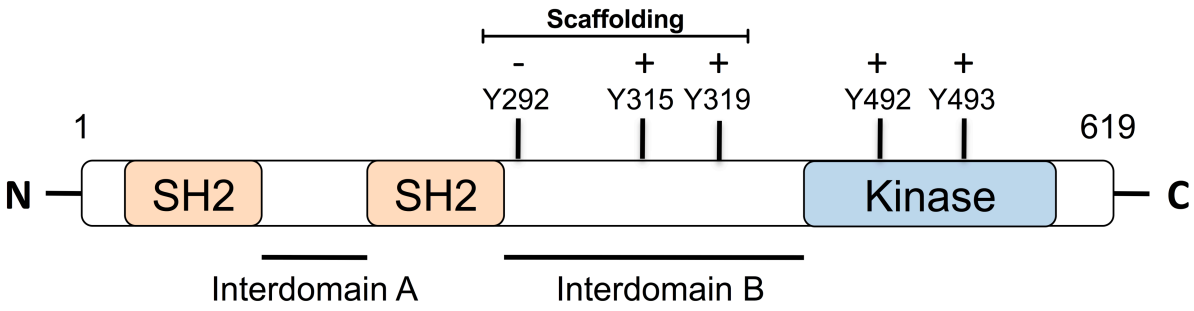

Scaffolding
-
+
+
+
+
Y292
Y315
Y319
Y492
Y493
1
619
N
SH2
SH2
Kinase
C
Interdomain A
Interdomain B

Figure 1.3 TCR signaling pathways

A schematic representation of signalling pathways activated downstream of the TCR, following signalling mediated by sp/pMHC. Crosslinking of the TCR and coreceptor induces the phosphorylation of ITAMs on CD3-ε, γ, δ and ζ-chains. Phosphorylated ITAMS facilitate binding and activation of SH2-domain containing proteins of which Zap70 is essential. The proximal TCR signalling events result in downstream phosphorylation of adaptor and scaffolding proteins including linker of activated T-cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (Slp76), thus inducing formation of a large signalling molecule complex. The associated effector molecule phospholipase C γ-isoform (PLCγ), hydrolyzes PIP_2 to Inositol triphosphate (IP_3) and diacylglycerol (DAG). These lipid products act as second messengers. The binding of IP_3 to IP_3-gated calcium channels induces store operated calcium entry and subsequently calcium influx. The cytosolic calcium binds calmodulin, which acts as a cofactor for the calcineurin enzyme. Calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), permitting nuclear translocation and transcriptional activity. In contrast DAG activates protein kinases including Ras guanyl nucleotide-exchanging protein (RasGRP) and PKC, through binding to C1 domains. This results in phosphorylation cascades, culminating in NFκB and activator protein-1 (AP-1) activation. Finally TCR signalling has been implicated in cytoskeletal reorganisation and cellular adhesion via Vav oncogene (Vav) and non-catalytic region of tyrosine kinase adaptor protein-1 (Nck) , which interact via the LAT scaffold. Transcription factors activated by TCR signalling are shown in shaded red.

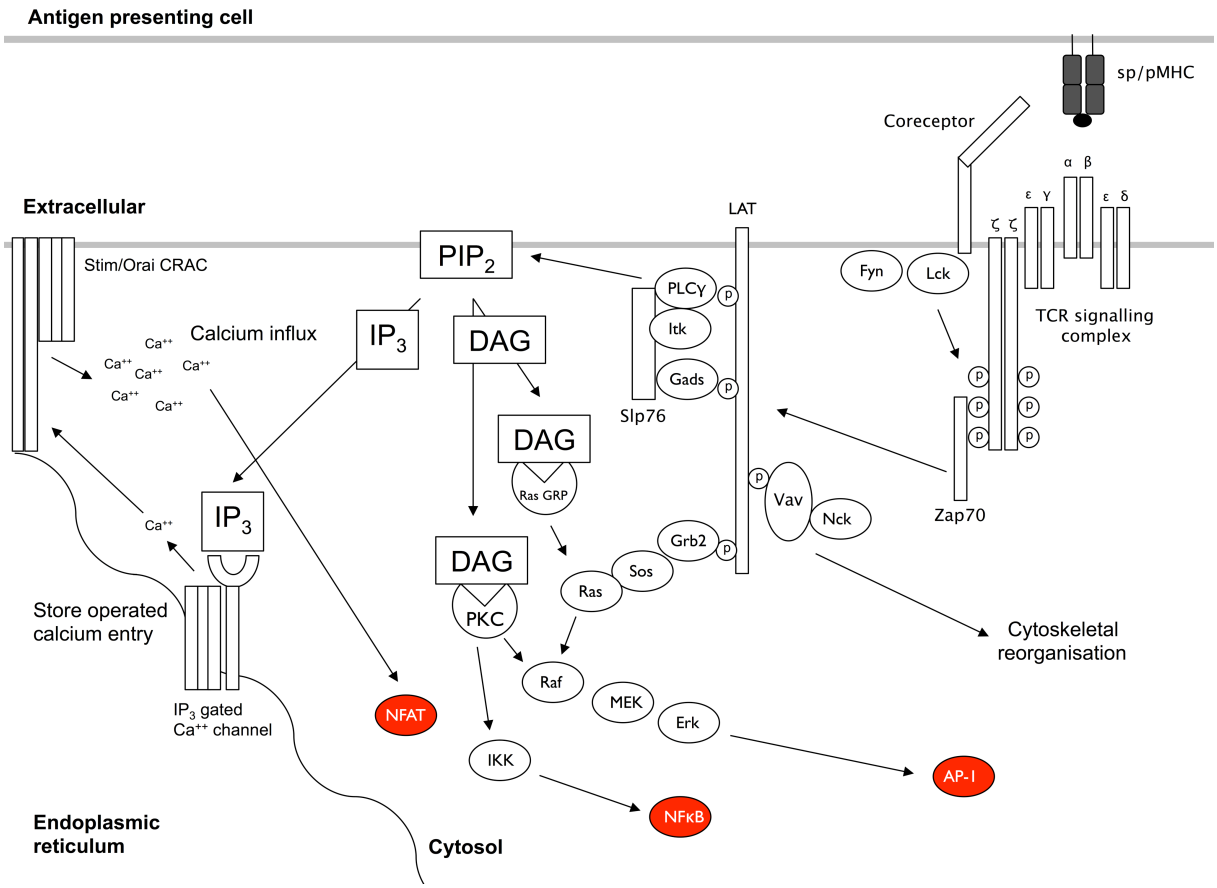
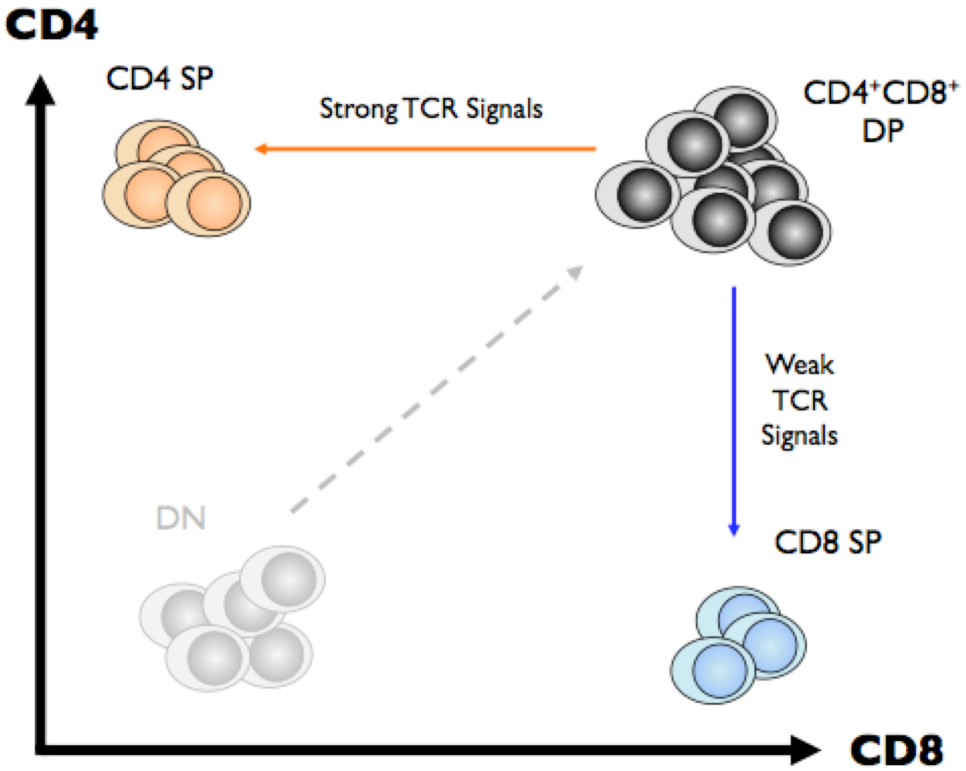

Antigen presenting cell
sp/pMHC
Coreceptor
Extracellular
LAT
Stim/Orai CRAC
PIP2
PLCγ
Itk
Gads
Slp76
Fyn
Lck
α β
ε γ
ζ ζ
ε δ
TCR signalling complex
Calcium influx
Ca++
IP3
DAG
Ras GRP
Zap70
Vav
Nck
Grb2
Sos
Ras
PKC
Raf
MEK
Erk
AP-1
Cytoskeletal reorganisation
Store operated calcium entry
IP3 gated Ca++ channel
NFAT
IKK
NFκB
Endoplasmic reticulum
Cytosol

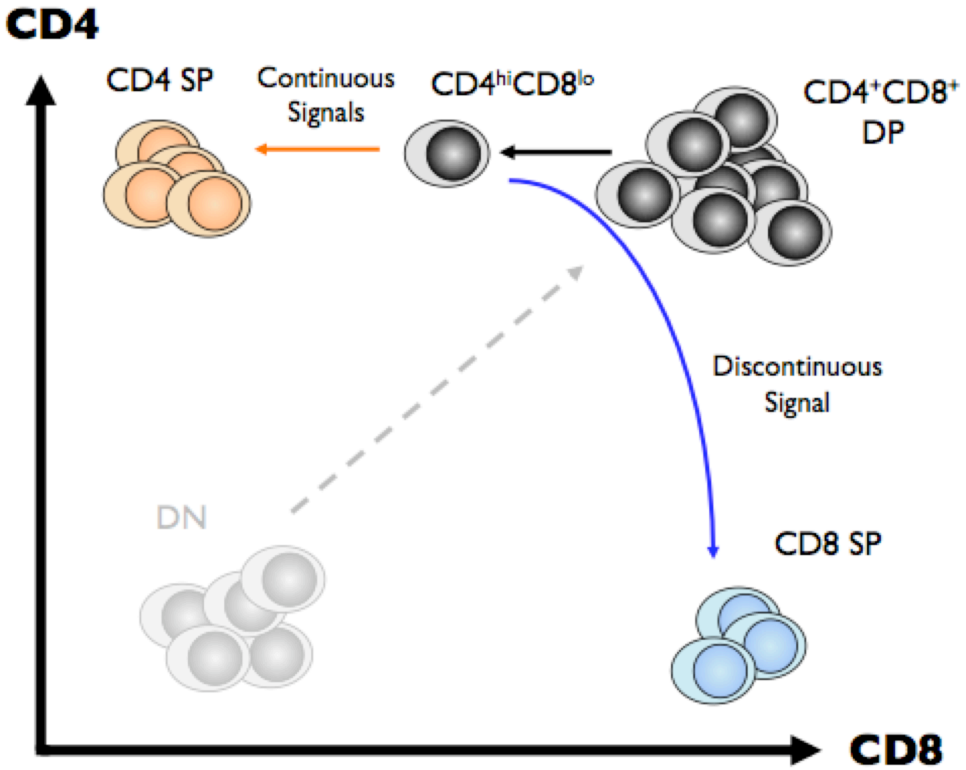
Figure 1.4 Instructive models of CD4/CD8 lineage commitment

A diagram showing the instructive models of lineage commitment. (A) The quantitative instructive model states that differences in signal strength underlie the CD4 versus CD8 lineage choice. Relatively stronger signalling mediated by CD4/TCR:spMHC instructs CD4 lineage commitment, whereas weaker signalling mediated by CD8/TCR:spMHC instructs CD8 lineage commitment. (B) The kinetic signalling model states that differences in signalling length underlie the CD4 versus CD8 lineage choice. Initial positive selection signalling induces transient downregulation of the CD8 coreceptor, so cells are phenotypically $CD4^{hi}CD8^{lo}$. Those cells receiving a consistent signal in the $CD4^{hi}CD8^{lo}$ populations are instructed to commit to the CD4 lineage, whereas the cells in which signalling is disrupted commit to the CD8 lineage. CD8 lineage commitment may possibly depend on additional instructive IL7 signals.

A **Quantitative instructive model**



B **Kinetic signalling model**



Chapter 2

Materials and Methods

2.1 Mice

2.1.1 Experimental mice used in this study

Mice used in this study are shown in **Table 2.1**. Mice were bred and housed at the NIMR animal facility under specific pathogen free (SPF) conditions in accordance with UK Home Office regulations. Genotyping was performed by a combination of flow cytometric analysis of blood and PCR analysis of DNA obtained from tails. All experimental mice were used at 5-12 weeks of age.

Table 2.1 List of mice used in this study.

Mouse strain (designated in text)	Background	Reference
BALB/c	BALB/c	
C57B6/NIMR (B6 CD45.2)	C57B6/NIMR	
B6.SJL-*Ptprc* (B6 CD45.1)	C57B6/J	
B6.129-$H2^{dlAb1\text{-}Ea}$ (MHC-II Δ/Δ)	C57B6/J	(Madsen *et al.*, 1999)
D011.10 $Rag1^{-/-}$	BALB/c	(Murphy *et al.*, 1990)
F5 $Rag1^{-/-}$ CD45.2 (F5 $Rag1^{-/-}$)	Mixed	(Mamalaki *et al.*, 1993)
F5 $Rag1^{-/-}$ CD45.1	Mixed	

F5$^{+/-}$ *Rag1*$^{-/-}$	Mixed	
F5$^{+/-}$ *Rag1*$^{-/-}$ *Zap70*$^{-/-}$ *Zap70*^{Tre} *rtTA*^{huCD2} (F5 TetZap70)	Mixed	(Saini *et al.*, 2010)
F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$	Mixed	(Smyth *et al.*, 1998)
F5$^{+/-}$ *Rag1*$^{-/-}$ *rtTA*^{HuCD2} *Zap70*$^{-/-}$ (F5$^{+/-}$ *Rag1*$^{-/-}$*Zap70*$^{-/-}$)	Mixed	(Saini *et al.*, 2010)
rtTA^{HuCD2} *Lck*$^{-/-}$ (*Lck*$^{-/-}$)	Mixed	(Molina *et al.*, 1992)
OT-I *Rag1*$^{-/-}$	C57B6/J	(Hogquist *et al.*, 1994)
OT-II *Rag1*$^{-/-}$	C57B6/J	(Barnden *et al.*, 1998)
Rag1$^{-/-}$	C57B6/J	(Mombaerts *et al.*, 1992b)
Rag1$^{+/-}$ *b2m*$^{-/-}$ (*b2m*$^{-/-}$)	Mixed	(Koller *et al.*, 1990)
Rag1$^{-/-}$ *b2m*$^{-/-}$	Mixed	
Rag2$^{-/-}$	C57B6/J	(Shinkai *et al.*, 1992)
SKG	BALB/c	(Sakaguchi *et al.*, 2003)
Zap70^{Tre} *rtTA*^{huCD2} *Zap70*$^{-/-}$ (TetZap70)	Mixed	(Saini *et al.*, 2010)
Zap70$^{-/-}$	C57B6/J	{Negishi, 1995 #39)

All C57B6 or mixed mice are on an H-2^{b} background, and all BALB/c mice are on an H-2^{d} background.

2.1.2 Tetracycline inducible Zap70 mice

Mice were generated bearing a construct encoding Zap70 and a human CD2 (huCD2) reporter gene, under control of a tetracycline response element (TRE) (*Zap70*^{Tre}) (Gossen & Bujard, 1992; Saini, 2007; Saini *et al.*, 2010) (TreZap70).

These mice were crossed with a strain expressing a reverse tetracycline transactivator protein (rtTA) under control of HuCD2 regulatory elements (*rtTA*^{huCD2}) (Legname *et al.*, 2000; Zhumabekov *et al.*, 1995). Mice were then bred to an endogenous Zap70 deficient background to generated *Zap70*^{Tre} *rtTA*^{huCD2} *Zap70*$^{-/-}$ mice (TetZap70) (**Figure 2.1**).

2.1.3 Doxycycline induction of TetZap70 mice

TetZap70 mice were induced to express Zap70 by the administration of the tetracycline derivative doxycycline (dox) in food, at a concentration of 3mg/g (3% w/w).

2.2 Media

Phosphate buffered saline (PBS) (was either produced in house at the NIMR or purchased from GIBCO).

Fluorescent activated cell sorting (FACS) buffer: PBS supplemented with either 5% (v/v) heat-inactivated fetal calf serum (FCS) (Biosera) or 0.5% (v/v) sodium azide (Sigma) and 0.5% (w/v) bovine serum albumin (BSA) (Sigma).

Air buffered Iscove's modified Dulbecco's medium (IMDM): produced in house at the NIMR.

Handling media: Air buffered IMDM containing 1% (w/v) BSA.

IMDM (FCS): Air buffered IMDM containing 5% (v/v) FCS.

ACK lysis buffer: 150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Ethylenediaminetetraacetic acid (EDTA).

Complete Dulbecco's modified Eagle's medium (cDMEM): Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% (v/v) FCS, 4mM L-glutamine (Sigma), 50μM β-mercaptoethanol (2ME) (Sigma), 100μg/mL Penicillin G sodium (Sigma) and 100μg/mL Streptomycin sulphate (Sigma).

Chemotaxis medium: Roswell Park Memorial Institute 1640 (RPMI) (GIBCO) supplemented with 5% (v/v) FCS, 0.5% (w/v) fatty acid (FA)-free BSA (Sigma),

4mM L-glutamine (Sigma), 100μg/mL Penicillin G sodium (Sigma) and 100μg/mL Streptomycin sulphate (Sigma).

Wash buffer (for confocal microscopy slides): PBS containing 1% FCS (v/v) 0.5% Tween20 (v/v) (Sigma).

2.3 Antibodies for flow cytometry

2.3.1 Primary antibodies

A list of primary antibodies used for flow cytometry in this study is shown in **Table 2.2**.

Table 2.2 List of conjugated cell surface antibodies used in this study

Specificity	Clone (manufacturer)	Conjugate/s	Working concentration
B220 (CD45R)	RA3-6B2 (eBioscience)	PE	1μg/mL
CD4	L3T4 (eBioscience)	PerCP, APC, PB, EF450, PO	1.25μg/mL
CD4	RM4-5 (Invitrogen)	PETR	1.25μg/mL
CD4	RM4-5 (Invitrogen)	PO	2.5μg/mL
CD5	53-7.3 (eBioscience)	FITC, PE-Cy7, APC, EF450	1.25μg/mL
CD8α	53-6.7 (eBioscience)	PE, PE-Cy7, EF450	0.5μg/mL
CD8α	5H10 (Invitrogen)	PETR	1μg/mL
CD8α	5H10 (Invitrogen)	PerCP, PO	0.5μg/mL
CD19	1D3 (eBioscience)	APC-Cy7	0.5μg/mL
CD24 (HSA)	M1/69 (eBioscience)	FITC	1.25μg/mL
CD25	PC61.5 (eBioscience)	PE-Cy7	1.25μg/mL
CD45.1	A20 (eBioscience)	FITC, PE	2.5μg/mL
CD45.2	104 (eBioscience)	PE, Biotin	2.5μg/mL
CD44	IM7 (eBioscience)	APC-EF780	0.5μg/mL
CD69	H1.2F3 (eBioscience)	PE	1μg/mL

CD127 (IL7rα)	A7R34 (eBioscience)	PE	1μg/mL
HuCD2	RPA-2.10 (BD)	PE	Diluted 1 in 50
HuCD2	RPA-2.10 (eBioscience)	PE-Cy5	Diluted 1 in 50
TCRβ	H57-597 (eBioscience)	PE, PeCy5, APC	1μg/mL

FITC – Fluoerescein isothiocyanate, PE – Phycoerythrin, PerCP – Peridinin-chlorophyll protein, PETR – PE-Texas Red, APC – Allophycocyanin, EF780 – eFluor780, EF450 – eFluor450, PB – Pacific Blue, PO – Pacific orange, HSA – Heat stable antigen, HuCD2 – Human CD2.

2.3.2 Intracellular antibodies

A list of Intracellular antibodies used in this study is shown in **Table 2.3**.

Table 2.3 List of intracellular antibodies used in this study

Antibody	Clone (Manufacturer)	Conjugate	Working Concentration
Erk2	G263-7 (BD)	APC-A647	Diluted 1 in 5
Fyn	Sc-434 (Santa Cruz Biotechnology)	PE	1μg/mL
LAT	Rabbit Polyclonal (Cell Signaling Technology)	n/a	Diluted 1 in 200
Lck	MOL 171 (BD)	n/a	1μg/mL
pSTAT5 (Y694)	47 (BD)	APC-A647	Diluted 1 in 50
Slp76	HS76 (eBioscience)	FITC	0.05μg/mL
Zap70	1E7.2 (eBioscience)	PE	1μg/mL

A647 – APC conjugated for Alexa-Fluor 647, Erk2 – Extracellular regulated kinase2, Fyn – Fyn proto-oncogene, LAT – Linker of activated T-cells, Lck – Leukocyte-specific protein tyrosine kinase, pSTAT – Phosphorylated signal transducer and activator of transcription, Slp76 – SH2 containing leukocyte protein of 76 kDa molecular weight, Zap70 – Zeta-chain-associated protein kinase of 70kDa molecular weight, kDa – kilodaltons, n/a – Not applicable (unconjugated antibody).

2.3.3 Secondary antibodies, reagents and isotype controls

A list of secondary antibodies, reagents and isotype controls used in this study are shown in **Table 2.4**

Table 2.4 List of secondary antibodies, reagents and isotype controls used in this study

Antibody/Reagent	Clone (Manufacturer)	Conjugate	Working Concentration
Human Fcγ (CCL19-Fc secondary)	Goat polyclonal (eBioscience)	PE	0.25μg/mL
Mouse IgG1 isotype control	A85- 1 (BD)	PE	1μg/mL
Mouse IgG1 isotype control	MOPC-21 (BD)	APC-A647	Diluted 1 in 5
Rabbit IgG1 (LAT secondary)	Goat Polyclonal (Jackson labs)	FITC	5μg/mL
Rat IgG2a isotype control	n/s (eBioscience)	FITC	2.5μg/mL
Streptavidin	n/a (eBioscience)	PE	0.5μg/mL
Streptavidin	n/a (BD)	PETR	0.5μg/mL

IgG – Immuglobulin G, Fcγ – Fc receptor γ-fragment

2.4 Preparation of single cell suspensions

2.4.1 Thymus, spleen and lymph nodes

Cervical, axillary, brachial, mesenteric and inguinal lymph nodes (LNs), spleen and thymus were dissected from mice. Lymphocytes were teased into ice cold handling media via mechanical disintegration through 75μm nylon mesh. Cells were washed 1-2 times with an equal volume of FACS buffer, centrifuged (Multifuge 3 s-r, Heraeus) (1200 RPM, 4min) and re-suspended in fresh FACS buffer. Total lymphocyte counts were taken using a Casy-1 cell counter (Schärfe System) according to the manufacturers instructions. Cells were kept on ice throughout.

2.4.2 Peripheral blood

100μL of peripheral blood was obtained by nicking the lateral tail vein of experimental mice with a scalpel blade. Blood was collected in microfuge tubes containing ~100μL heparin (dissolved in PBS, according to manufacturer's instructions, Sigma). Red blood cell lysis was performed by the addition of 2mL ACK lysis buffer followed by vigorous vortexing, for approximately three minutes at room temperature, before samples were washed in 2mL ice cold FACS buffer.

2.5 Flow cytometry

2.5.1 Surface immunostaining and FACS analysis

Lymphocytes were incubated on ice and in the dark in 100μL primary antibody cocktail per 2-5x10^6 cells, made with antibodies conjugated to fluorescent labels or biotin. Antibodies were made up to appropriate working concentrations (indicated in **Table 2.2**) in ice-cold FACS buffer and staining was performed for one hour. To visualise biotinylated antibodies, cells were washed once and secondary staining was performed with streptavidin conjugated to PE or PETR (as detailed in **Table 2.4**). Cells were then washed 1-2 times in FACS buffer and filtered through 35μm-pore cell strainers (BD Falcon). Flow cytometry was performed on CyAN ADP (Beckman Coulter), BD LSRII, BD FACSCantoII or BD FACSAria flow cytometers (all BD) and data analysis was performed using Flowjo software (v8.8.6 or v9.01) (Tree Star).

2.5.2 CCR7 detection

Lymphocytes were incubated on ice and in the dark, in primary antibody cocktails additionally containing CCL19-Human Fc receptor γ-fragment (Fcγ) (eBioscience) at a working concentration of 0.25μg/mL. Cells were stained in 100μl of antibody cocktail per 2x10^6 cells, for one hour. Lymphocytes were then washed one time, and subsequently incubated in 100μL/2x10^6 cells PBS containing 2% normal rat serum (Caltag), 2% Normal mouse serum (Invitrogen) and PE-conjugated anti-human IgG Fcγ fragment specific mouse polyclonal

antibody (**Table 2.4**) for one hour. Cells were subsequently washed 1-2 times and analysed by flow cytometry.

2.5.3 Intracellular flow cytometry

2.5.3.1 NP40 permeablisation

Cells were first stained for surface antigens as described in section **2.5.1**. For analysis of Fyn, Lck or Zap70 expression, cells were washed once in PBS and fixed with IC fixation buffer (eBioscience) at room temperature for 20 minutes. Cells were washed and re-suspended in 0.1% nonidet P-40 (NP40) (Igepal ca-630) (Sigma-Aldrich) in distilled water (dH_2O) (v/v) for three minutes. Cells were subsequently washed in 1mL PBS and re-suspended in intracellular antibody cocktail containing antibody at concentrations indicated in **Table 2.3**, made up in PBS. Staining was performed overnight. For unconjugated antibodies (Lck), cells were washed and primary staining was visualised following a secondary incubation with polyclonal anti-mouse IgG1 conjugated to PE at appropriate concentrations in 100μL per $2x10^6$ cells, for one hour (**Table 2.4**). Cells were washed twice and analysed by flow cytometry. Where applicable, cells stained with isotype control antibodies were treated in an identical manner.

2.5.3.2 Methanol Permeablisation

For analysis of Erk2, LAT, pSTAT5 and Slp76 expression, lymphocytes were surface stained, washed once in PBS and fixed in IC fixation buffer for 20 minutes at room temperature. Cells were washed and re-suspended on ice with

500μL 90% methanol in dH_2O (v/v) (originally at -20^oC) for 28 minutes. Lymphocytes were the washed twice in at least 4mL FACS buffer, before being incubated with intracellular antibody at appropriate concentrations (**Table 2.3**), in 100μL FACS buffer for one hour. Staining was performed on ice, with the exception of pSTAT5, where it was performed at room temperature. For unconjugated antibodies (LAT), cells were washed and primary staining was visualised following a secondary incubation with polyclonal goat anti-rabbit IgG1 conjugated to FITC at appropriate concentrations in 100μL per $2x10^6$ cells, for one hour (**Table 2.4)**. Cells were then washed twice and analysed by flow cytometry. Where applicable, cells stained with isotype control antibodies were treated in an identical manner.

2.6 Radiation bone marrow chimeras

2.6.1 Bone marrow isolation

Bone marrow (BM) was isolated from tibiae and femora of TetZap70 mice. Epiphyses were removed and BM was obtained by flushing handling media through the medullary canal with a 25-gauge needle. The BM was next filtered through a 35μm cell strainer and washed twice in handling media before being re-suspended in handling media at a concentration of ~$2.5x10^7$ cells/mL.

2.6.2 Irradiation and bone marrow reconstitution

Rag1$^{-/-}$ host mice were irradiated with 500 Rads (caesium source) and allowed to rest for 12-24 hours. Transfer of BM cells was performed by intravenous

(I.V.) administration of ~200μL cell suspension (5x10^6 cells) into the lateral tail vein of irradiated host mice with a 0.5mL microfine insulin syringe (BD). Mice were subsequently treated with water supplemented with 0.02% (v/v) Baytril® for one month (Sigma, UK). Mice were left for at least eight weeks to allow reconstitution of the lymphoid compartment, which was confirmed by phenotypic analysis of peripheral blood. Peripheral blood was prepared as described in section **2.3.2**. Cells were washed with a further 2mL FACS buffer and analysed for the expression of cell surface B220, CD19 and TCR by flow cytometry as described in section **2.5.1**. Reconstituted chimeras were identified by the presence of B220$^+$CD19$^+$ B-cells and the absence of T-cells.

2.7 Cell sorting

Cells were stained for expression of cell surface markers as described in section **2.5.1**, with handing media used in place of FACS buffer. Cells were then washed and re-suspended in handling media at a concentration of ≤1x10^8 cells/mL. FACS sorting was performed using Moflo (Dako) or BD FACSAria (BD) cell sorters, and sorted cells were collected in ice cold handling media.

2.8 mRNA expression analysis

2.8.1 Isolation of total cellular mRNA

Cell populations were obtained by cell sorting (section **2.6**), or total lymph node T-cells (LNTs) were used (as indicated in figure legends). Cells were washed in PBS and were lysed in 750μL Trizol® reagent (Invitrogen), in sterile 1.5mL

microfuge tubes (Eppendorf) at a concentration of ≤7.5x10^6 cells/mL. Samples were mixed by repetitive pipetting and incubated at room temperature for ~10 minutes. 200μL of phenol-chloroform was added to samples, which were shaken vigorously and left for 5-10 minutes to permit phase separation. Samples were centrifuged at 12000RPM for 15 minutes at 4°C in a mini-centrifuge (Biofuge *fresco*, Heraeus). The aqueous phase was transferred into a fresh sterile microfuge tube with an equal volume of isopropanol. Samples were snap frozen on dry ice and stored overnight at -70°C. Samples were thawed and centrifuged in a mini-centrifuge at 13000 RPM for 30 minutes, before the supernatent was carefully removed by pipetting. Pellets were washed once in 200μL 70% ethanol in dH_2O (v/v) and centrifuged for five minutes at 13000 RPM. The RNA pellet was re-suspended in 20μL RNase free dH_2O (Ambion) and RNA was quantified by a nanodrop 1000 (Thermoscientific).

2.8.2 Reverse transcription of mRNA to cDNA

First strand cDNA synthesis was produced using olio$(dT)_{12\text{-}18}$ primers (Invitrogen). cDNA was produced with Superscript II reverse transcriptase (Invitrogen). Both were used according to the manufacturer's instructions.

2.8.3 Quantitative real time polymerase chain reaction

PCR mix was made with 12.5μL/reaction TaqMan universal PCR master mix (Applied Biosystems), 1.25μL/reaction qPCR assay mix (Applied Biosystems) containing PCR primers (900nM final concentration), 6-carboxyfluorescien (FAM) labelled TaqMan MGB probe (250nM final concentration) and 1.25μL

RNase free dH_2O. PCR primers used in this study are shown in **Table 2.5**. PCR mix (15μL/reaction) was added along with 10μL cDNA sample (containing 40ng of RNA prior to reverse transcription, diluted in dH_2O) into one well on a MicroAmp 96-well reaction plate (Applied Biosystems). All reactions were performed in triplicate for 45 cycles on an ABI x7900 or x7000 Sequence Analyser (Applied Biosystems), following the manufacturer's instructions. Data was analysed by the standard curve method (Applied Biosystems) (reviewed in (Nolan *et al.*, 2006)) and expression of the gene of interest was normalised to expression of the housekeeping gene *Hypoxanthine-guanine phosphoribosyltransferase1* (*Hprt1*) (Steen *et al.*, 1990). Normalised expression was calculated according to the equation:

$$\text{Normalised expression} = \frac{2\text{^}(-\Delta Ct_{Hprt1})}{2\text{^}(-\Delta Ct_{\text{Gene of interest}})}$$

Ct is an arbitrary measure of fluorescence and is the mean average of three replicate wells.

Table 2.5 qPCR primers used in this study

Common Name	**Gene**	**mRNA designation in text**	**Applied Biosystems primer-probe assay number**
CD5	*Cd5*	Cd5	Mm00432417_m1
Cathepsin W	*CtsW*	CtsW	Mm00515599_m1

Forkhead box O1	*Foxo1*	Foxo1	Mm00490672_m1
GA binding protein transcription factor, beta subunit 1	*Gabpb1*	Gabpb1	Mm00487471_m1
GATA binding protein 3	*Gata3*	Gata3	Mm01337569_m1
Hypoxanthine-guanine phosphoribosyltransferase	*Hprt1*	Hprt1	Mm01318743_m1
Interleukin-7 receptor α-chain	*IL7r*	Il7r	Mm00434295_m1
Runt-related transcription factor 1	*Runx1*	Runx1	Mm01213405_m1
Runt-related transcription factor 1	*Runx3*	Runx3	Mm00490666_m1
Zinc finger and BTB domain containing 7B	*Zbtb7b*	Thpok	Mm00784709_s1
Zeta-chain-associated protein kinase of 70kDa molecular weight	*Zap70*	Zap70	Mm00495255_m1

2.9 Intrathymic cell transfers

Donor cells were isolated by cell sorting as described in section **2.7**, or F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymi were used as a source of non-selecting F5 donor DP thymocytes. Cells were re-suspended at a concentration of ≤1x10^{8} cells/mL in handling media. Intrathymic injection was performed with modifications from a previously published protocol (Goldschneider *et al.*, 1986). Mice were anaesthetised through inhalation of isofluorane (Abbott). A ~1-2cm incision was made in the skin, along the midline overlying the lower cervical and upper thoracic region. Cell suspension (10μL, 5x10^{4}-1x10^{6} cells) was injected into the anterior superior portion of each thymic lobe using a 1mL microfine insulin syringe mounted on a Tridak stepper (Tridak). Injection depth varied from

~5-10mm. Following injection, the incision was closed with 9mm surgical wound clips (BD). Mice were left for indicated numbers of days, before entire thymi were analysed by flow cytometry for expression of cell surface and intracellular antigens as described in section **2.5**. Flow cytometric analysis of whole thymi was performed using a CyAN ADP flow cytometer.

2.10 Cell culturing

2.10.1 One day culture of DP thymocytes

Thymocyte subpopulations were obtained by cell sorting (section **2.7**) and cultured at 37°C in a Sanyo CO_2 incubator (Sanyo) at 5% carbon dioxide (CO_2) overnight in cDMEM in 96 well plates, in 100μL medium at a concentration of $\leq 5x10^6$ cells/mL. Cells were then washed once before analysis of cell surface marker expression by flow cytometry as described in section **2.5**.

2.10.2 *in vitro* positive selection culture

Non-selecting (DP1, see **chapter 3**) thymocytes were obtained through the negative selection of mature thymocytes from B6 thymus. B6 thymocytes were surface stained at a concentration of $\leq 1x10^8$ cells/mL with biotinylated antibodies to CD3 CD25 and CD69 (1μg/mL CD3 (clone 145-2C11, eBioscience), 2.5μg/mL CD25 (clone PC61.5, eBioscience) and 2.5μg/mL CD69 (clone 17A2 BD)). Cells were washed and depleted using streptavidin coated Dynal® magnetic beads (Invitrogen) at a 1:1 ratio to cells, at a concentration of

2-4x10^7 cells/mL according to manufacturer's instructions. Unlabelled cells were collected, transferred to a fresh tube and counted.

in vitro positive selection cultures were based on a previously published protocol (Ohoka *et al.*, 1996) . Lymphocytes (4-5x10^6) were cultured in 1mL cDMEM with Phorbol 12-myristate 13-acetate (PMA) (0.2ng/mL) (Sigma) and ionomycin (0.2μg/mL) (Sigma) for 14 hours at 37°C in 5% CO_2 (primary culture) unless otherwise indicated. Cells were washed twice in fresh cDMEM and cultured in 1mL cDMEM for the remaining time as indicated (secondary culture = total culture time less 14 hours primary stimulation). Where indicated, inhibitors were used throughout the primary and secondary cultures at the following concentrations: actinomycin D (ActD) (5μg/mL), cyclohexamide (Chx) (1μg/mL), Kn93 (20ng/mL), U0126 (3.8μg/mL), cyclosporin A (CsA) at indicated concentrations (1-100ng/mL) and FK506 (10ng/mL) (all Sigma). Cells were harvested at the timepoint indicated and analysed by flow cytometry (section **2.5**).

2.11 Chemotaxis assays

Thymocytes were dissected into chemotaxis medium, counted and re-suspended in chemotaxis medium at a concentration of 1x10^8 cells/mL. Chemotaxis assays were performed in 24-well tissue culture transwell plates with 5μm pore polycarbonate filters (Corning). Transwell inserts (upper chamber) were coated overnight at 37°C with 100μL 2μg/mL fibronectin in PBS (R&D systems). Fibronectin was then replaced with 100μL 1% FA-free BSA in

PBS and incubated at 37ºC for one hour. The 1% FA-free BSA solution was then replaced with 100μL of cell suspension (1x10^7 cells/well). 600μL CCL21 chemokine (R&D systems) was added to the lower chamber at a concentration of 500ng/mL. Cells were incubated at 37°C with 5% CO_2 for four hours to permit chemotaxis. As an input control, 1x10^7 cells were added directly to the lower chamber of a control well with identical amounts of chemokine. Following incubation, transwell inserts were discarded and FITC conjugated polystyrene beads (caliBRITE beads, BD) were added to samples and control wells. Beads were diluted (~3 drops in 5mL) and 100μL beads were added per well. Cells were washed in FACS buffer and underwent surface staining as described in section **2.5.1**. Cells were then analysed on a BD LSRII flow cytometer. Beads could be distinguished from cells based on their higher side scatter (SSc) relative to lymphocytes. Frequency of migration (% migration) for gated cell populations was calculated according to the following equation:

$$\% \text{ migration} = \frac{(\text{Total beads/gated lymphocytes})_{\text{Experimental well}}}{(\text{Total beads/gated lymphocytes})_{\text{Control well}}} \text{ x100}$$

2.12 Confocal microscopy

2.12.1 Embedding, sectioning and fixation of tissue

Thymi were dissected, snap-frozen on blocks of dry ice and stored at -70°C until use. Wet filter paper was frozen onto a chuck inside a cryostat (Bright Instrument Co Ltd). Optimal cutting temperature (OCT) compound (1mL) was

next used to adhere whole thymi onto the filter paper before being frozen with Cryospray (Bright Instrument Co Ltd.). Continuous 20μm slices were made with a microtome (Bright Instrument Co Ltd) to remove epithelial layers and expose the internal thymic structure. 5μm sections were cut and adhered to a four spot Vectabond coated multispot slide (Vector Laboratories). Slides were dried at room temperature for at least one hour and fixed in pre-cooled 90% acetone (v/v) at 4°C for 20 minutes. Following fixation slides were air dried for 10 minutes and stored at -20°C.

2.12.2 Immunolabelling and visualisation of tissue sections

Slides prepared as above (section **2.12.1**) were washed in wash buffer in a glass slide holder. Slide spots were then dried with grade-three filter paper (Whatman) and placed in a humid chamber for incubation. Antibody staining was then performed for one hour at room temperature. Slide spots were incubated with 50μL antibody cocktail containing antibodies specific for CD4 (clone GK1.5, eBioscience) conjugated to Alexa-Fluor 647 with an antibody labelling kit (Molecular probes)) and CD8-Bio (clone H3517.2, eBioscience). Following a 15 minute wash in wash buffer, visualisation of CD8-Bio was performed by a secondary incubation with streptavidin Alexa-Fluor 555 (Invitrogen). Slides were washed for 15 minutes in wash buffer and airtight coverslips were sealed on with clear nail varnish (La Femme). Visualisation was performed using a Zeiss Confocal Microscope (Carl Zeiss AG).

2.13 Analysis of intracellular calcium flux by flow cytometry

Isolated lymphocytes were re-suspended in IMDM (FCS), counted and incubated at a concentration of 4-6x10^6 cells/mL with the calcium indicator Indo1 (2μg/mL) (Grynkiewicz *et al.*, 1985), conjugated to acetoxymethyl ester (Indo1 AM) (Invitrogen). Cells were incubated for 40 minutes at 37ºC to facilitate hydrolysis of Indo1 AM to Indo1 by endogenous esterases. Cells were washed and incubated at room temperature for 30 minutes in antibody cocktails, made up in IMDM (FCS) and additionally containing 10μg/mL anti-CD3 (clone 145-2C11, BD). Cells were washed and re-suspended in ~300μL handling media at room temperature, for use as a stock sample. Stock sample was diluted 1 in 10 in room temperature handling media and analysed on a BD LSRII flow cytometer, equipped with a 355nm UV laser, at a rate of ~20,000 events/second. Analysis was performed for 30 seconds, before addition of 0.2μg/mL crosslinking antibody (polyclonal goat anti-Armenian hamster IgG (H&L)) (Rockland) or 1μg/mL ionomycin. Samples were vortexed briefly, before being analysed for a further ~10 minutes. Data was analysed using Flojo V8.8.6. Cells were analysed for the ratio of Violet (405±20nm, calcium bound):Blue (530±30nm, calcium unbound) light emission over time. Calcium flux was shown graphically as the percentage of cells over an arbitrary threshold of Violet/Blue emission (kept consistent throughout the same experiment) as a moving average over time.

2.14 Preparation of cell lysates and western blotting

2.14.1 Western blotting reagents and solutions

2.14.1.1 Solutions

Lysis buffer: 50mM tris pH7.5, 150mM NaCl, 1mM NaOVa, 5mM EDTA, 10mM NaF, 10mM sodium pyrophosphate, 1% NP40 and one complete mini protease inhibitor cocktail (Roche).

Sample buffer: 187.5mM tris-HCL, 6% (w/v) sodium dodecyl sulphate (SDS) (Sigma), 30% (w/v) glycerol, 150mM dithiothreitol (DTT) and 0.03% (v/v) bromophenol blue.

PBS-Tween: PBS and 0.001% (v/v) Tween20.

Running buffer (Bio-Rad): 10x stock (tris/glycine/SDS buffer) diluted in dH_2O.

Transfer buffer: 10mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer pH11.

Membrane blocking solution: PBS with additional 3% (w/v) skimmed milk powder (Marvel) and 0.05% Tween20 (v/v).

2.14.1.2 SDS-PAGE acrylamide protein gels

10% resolving gel: 1M Tris at pH8.8, H_2O, 10mL protogel (National Diagnostics), 300mL 20% (w/v) SDS, 100μL 10% (w/v) ammonium persulphate (AMPS), 20μL Tetramethylethylenediamine (TEMED).

4% Stacking gel: 2.5mL 1M Tris at pH6.8, 13.3mL H_2O, 3.3mL protogel, 200mL 20% (w/v) SDS, 100mL 10% (w/v) AMPS, 20mL TEMED (10mL volume). The stacking gel was cast over the resolving gel to aid sample resolution.

2.14.2 Preparation of cell lysates

Cell populations were obtained by cell sorting as described in section **2.6**. Cells were lysed at a concentration of $1x10^7$ cells/mL in lysis buffer for 30 minutes at 4°C. Lysates were centrifuged at 13000 RPM for 30 minutes at 4°C in a mini-centrifuge to pellet cellular debris. Supernatant was decanted into a new microfuge tube and incubated with an equal volume of sample buffer and agitated for one hour or overnight at 4°C. Samples were stored at -70°C until use.

2.14.3 Western blotting

Samples were heated to 100°C for five minutes and spun for four minutes at 13000 RPM in a mini-centrifuge. Lysate equivalent to $2x10^5$ cells was loaded onto the stacking gel in a volume of 75μL and was separated on a 10% SDS-PAGE gel in running buffer overnight (~14 hours) at 50V. Control lanes were loaded with Rainbow coloured protein molecular weight marker (Amersham Biosciences). Proteins were wet transferred onto methanol activated polyvinylidene difluoride (PVDF) membranes (Millipore) in 10mM transfer buffer at ~400mA for 2.5 hours. Membranes were blocked in membrane blocking solution for one hour at room temperature on a platform rocker (Bibby Scientific). Membranes were washed 3x15 minutes in

PBS-Tween and incubated with 50mL monoclonal Zap70 antibody (diluted 1 in 1000 in PBS-Tween, clone 99F2, Cell Signaling Technology) or 50mL monoclonal GAPDH control (diluted 1 in 5000 in PBS-Tween, clone 14C10, Cell Signaling Technology) overnight on a platform rocker at 4°C. Membranes were then washed (3x15 minutes) in PBS-Tween at room temperature and Horseradish peroxidase (HRP) linked secondary antibody (Anti-goat IgG) (Promega) diluted 1 in 5000 in 1% (w/v) skimmed milk powder in PBS-Tween on a platform rocker for one hour. Protein bands were visualised by chemiluminescence with enhanced chemiluminescence reagent (ECL) (Amersham) developed on medical X-ray film (Kodak).

2.14.4 Quantification of protein expression

Zap70 protein expression was normalised to GAPDH using densitometric analysis of scanned blots, with Image SXM software v1.62 (NIH).

2.15 Comparative genomics

Comparative genomics of the *Zap70* 5' and upstream region and graphical annotation were performed using ECR browser, available online at http://ecrbrowser.dcode.org (accessed September 2010). Evolutionary conserved transcription factor binding sites were identified using rVISTA 2.0 (Loots & Ovcharenko, 2004), via the ECR browser website, utilising the TRANSFAC professional V10.2 library for vertebrates, with matrix similarity optimized for function. Mouse versus human alignment of sequence was performed using the ECR transcription factor binding site alignment function.

2.16 Adoptive transfer of F5 and OT-I lymph node cells

2.16.1 Intravenous adoptive cell transfers

Lymphocytes were obtained from LN of OT-I *Rag1*$^{-/-}$ or F5 *Rag1*$^{-/-}$ mice as described in section **2.4**, before being re-suspended at a concentration of 2.5x10^{7}/mL. Cell suspension (200μL, 5x10^{6} cells) was administered intravenously into the lateral tail vein of indicated hosts, using a 0.5mL microfine insulin syringe. Mice were left until indicated timepoints following injection, before phenotypic analysis of T-cells was performed by flow cytometry as described in section **2.5**.

2.16.2 pSTAT5 induction

Lymphocytes were transferred, re-isolated and surface stained as described in section **2.16.1**. Where <5x10^{6} cells were isolated, tubes were spiked with congenically disparate B6 T-cells to ensure equal staining. Samples were washed in 1mL PBS, prewarmed to 37°C and stimulated with indicated amounts of interleukin (IL)-7 (Peprotech) for 30 minutes at 37°C. Fixation was performed by addition of an equal volume of IC fixation buffer to samples after 30 minutes, before incubation at room temperature for 30 minutes. Samples were subsequently permeablised with methanol and stained for intracellular pSTAT5 (section **2.5.3**). Flow cytometry was performed on a BD FACSCantoII or BD LSRII flow cytometer and analysed using Flojo software (V8.8.6-9.0.1).

2.17 Calculations and statistical testing

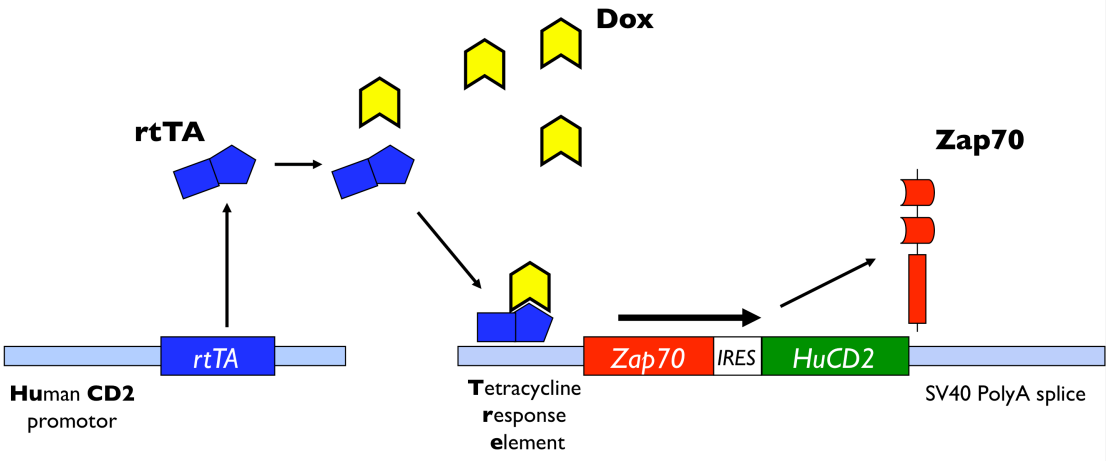
Calculations were performed using Microsoft Excel for Mac V12.2.4 (Microsoft). Statistical tests (two-tailed small sample t-tests unless otherwise indicated) were performed using Prism 5 for Mac OS X (Graphpad).

2.18 Collaborations

Flow cytometry for intact TetZap70 mice in **Figure 3.2 D** and B6 and TetZap70 mice in **Figure 5.1 F-H** was performed by Moni Saini and Ben Seddon at NIMR, UK. Jara Joedicke performed chemotaxis assays in **Figure 3.10 C**, at NIMR, UK. Confocal microscopy and slide preparation in **Figure 3.11** was performed in collaboration with Dr. Andrea White at the University of Birmingham, UK.

Figure 2.1 Tetracycline inducible control of Zap70 expression

Illustrative diagram showing doxycycline (dox) inducible control of the Zap70 and HuCD2 transgenes. TetZap70 mice express the reverse tetracycline transativator (rtTA) (blue) under control of the HuCD2 promotor (*rtTA*^{HuCD2}) and Zap70/HuCD2 reporter under control of a tetracycline response element (*Zap70*^{Tre}). In the presence of dox (yellow), the rtTA protein binds the tetracycline response element, where it acts as a transcription factor, driving the expression of *Zap70* (red) and *HuCD2* (green). TetZap70 mice are on an endogenous Zap70-deficient background, ensuring that the only source of Zap70 come from this tetracycline inducible system.


Dox
rtTA
Zap70
rtTA
Zap70
IRES
HuCD2
Human CD2
promotor
Tetracycline
response
element
SV40 PolyA splice

Chapter 3

The Kinetic Regulation of Positive Selection and Lineage Commitment

3.1 Introduction

The thymic development of $\alpha\beta$-T-cells is a requirement for a functional immune system. Patients with defects in T-cell development (Arpaia *et al.*, 1994; Elder *et al.*, 1994; Gelfand *et al.*, 1995) or in ontogeny of the thymus (Kirkpatrick & DiGeorge, 1968) typically manifest a severe combined immunodeficient (SCID) phenotype. Therefore the mechanisms governing T-cell development and differentiation are of interest with regards to developing future treatments. Classically developmentally intermediate populations of thymocytes have been characterised by the phenotypic changes in a few surface proteins. Different maturation stages can be identified based on the expression of the T-cell receptor (TCR) coreceptors CD4 and CD8. Thymocytes are initially double negative (DN), lacking expression of the CD4 and CD8 coreceptors but can be further subdivided based on the expression of CD44 and CD25 ($CD44^+CD25^-$; DN1, $CD44^+CD25^+$; DN2, $CD44^-CD25^+$; DN3 and $CD44^-CD25^-$ DN4) (Godfrey *et al.*, 1993). T-cells subsequently enter a $CD4^+CD8^+$ double positive (DP) stage, before they commit to either the CD4 or CD8 single positive (SP) lineage. The ability to phenotypically distinguish between developmentally intermediate

populations of thymocytes has enabled the elucidation of important developmental checkpoints throughout T-cell ontogeny.

Progression through the DN stages depends on successful rearrangement and expression of the TCRβ chain, pairing with an invariant alpha chain (pre-Tα) and subsequent pre-TCR signalling at the DN3 stage (known as β-selection) (Godfrey *et al.*, 1994; Mombaerts *et al.*, 1992a). β-selection is followed by rearrangement and expression of the TCRα chain (Koyasu *et al.*, 1997), which is expressed along with both CD4 and CD8 coreceptors at the DP stage (Levelt *et al.*, 1993). αβ-TCR signalling is required for further development of DP thymocytes and is mediated by contact with self peptide antigen displayed in the context of major histocompatibility complex (spMHC) ligands on cortical thymic epithelial cells (cTECs) (Mombaerts *et al.*, 1992a)(reviewed in (Starr *et al.*, 2003)). Only those T-cell clones that weakly recognise spMHC will mature beyond the DP stage, through a process known as positive selection. As a further level of complexity those T-cell clones recognising spMHC-II (which additionally depends on CD4 coreceptor recognition) will subsequently commit to the CD4 SP lineage, whereas T-cell clones recognising spMHC-I molecules (additionally depending on CD8 coreceptor recognition) will commit to the CD8 SP lineage. The mechanisms underlying how a TCR signal can result in divergent developmental outcomes based on its major histocompatibility complex (MHC)-restriction have been the subject of much research (reviewed in (Germain, 2002)).

Positive selection TCR signalling depends on transduction through downstream kinases. The most proximal signalling event is the recruitment of the kinase leukocyte-specific protein tyrosine kinase (Lck) to the TCR signalling complex, which depends on the cytoplasmic tails of either CD4 or CD8 (Rudd *et al.*, 1988; Veillette *et al.*, 1988). Following initiation of TCR signalling, Lck activates Zeta-chain-associated protein kinase of 70Kda molecular weight (Zap70) (Iwashima *et al.*, 1994) and Zap70 itself targets a number of downstream adaptor and effector proteins, which leads to positive selection (reviewed in (Samelson, 2002)). Indeed *Zap70*$^{-/-}$ mice show developmental arrest at the pre-positive selection DP stage, showing an essential role for Zap70 in transducing the positive selection signal (Negishi *et al.*, 1995). In contrast, how a positively selecting cell distinguishes an MHC-I versus MHC-II mediated selection signal is less well established.

A currently favoured kinetic signalling model proposes that following an initial non-lineage-specifying signal, DP cells transiently downregulate expression of the CD8 coreceptor and enter a $CD4^{hi}CD8^{lo}$ population. It is suggested that CD4 dependent MHC-II signals can persist, whereas CD8 dependent MHC-I signalling is broken off. This led to the hypothesis that interpretation of a continued or intermittent TCR signal underlies the CD4 versus CD8 fate decision (Brugnera *et al.*, 2000; Yasutomo *et al.*, 2000). Nevertheless, whether the $CD4^{hi}CD8^{lo}$ population is indeed an obligate step for CD8 lineage commitment is controversial (reviewed in (Hogquist, 2001)). In addition, neither of these models addresses whether the CD4 versus CD8 lineage decision is

temporally regulated, as is commonly the case for developmental fate decisions (reviewed in (Freeman & Gurdon, 2002)).

A number of recent studies have addressed the question of how lineage fate is specified by investigating the downstream factors responsible for lineage fixation. For example, the nuclear factors GATA-binding protein 3 (Gata3) and T-helper-inducing *POZ*/Kruppel-like factor (ThPOK) have been shown to be essential for CD4 SP lineage commitment (He *et al.*, 2005; Hernandez-Hoyos *et al.*, 2003; Pai *et al.*, 2003). In contrast Runt-related transcription factor (Runx)3 is required for CD4 silencing and CD8 SP lineage development (Egawa *et al.*, 2007; Taniuchi *et al.*, 2002). Importantly, whilst many transcription factors involved in lineage commitment are known, the question remains as to how their expression is related to the upstream positive selection signal. Although it is known that positive selection occurs over a number of days (Lucas *et al.*, 1993; Lucas *et al.*, 1994; Lucas & Germain, 1996; Lundberg & Shortman, 1994), there are a lack of well-characterised phenotypic markers that can distinguish populations of developmentally intermediate cells. This has proved to be a major obstacle with regards to answering the question of how TCR signalling directs lineage fate.

Following lineage commitment SP T-cells migrate from the thymic cortex to the medulla. In the medulla they are exposed to peripheral tissue restricted antigens (TRAs), which if strongly recognised by a T-cell clone will induce clonal deletion (referred to as negative selection) (reviewed in (Anderson *et al.*, 2007)). If T-cells can avoid negative selection, they emigrate from the thymus, populate

peripheral lymphoid tissues and are henceforth competent to elicit an immune response (reviewed in (Starr *et al.*, 2003)). Recently thymic emigration has been tracked in mice expression GFP under control of the *Rag2* promotor. These experiments have suggested that medullary residency time is as short as 4-5 days (McCaughtry *et al.*, 2007). These results are in contrast to older experiments suggesting a longer thymic residency time (Egerton *et al.*, 1990). However, the kinetics of migration from the cortex to the medulla following positive selection have not been studied. For example it is unknown whether this migration differs for cells recognising MHC-I versus MHC-II.

In this chapter we have sought to investigate the temporal regulation of positive selection and the phenotypic changes associated with CD4 versus CD8 lineage commitment. Furthermore, we wanted to investigate the kinetics of SP thymocyte maturation, as cells migrate to the thymic medulla and undergo peripheral export. *Zap70*$^{-/-}$ mice are blocked at the DP stage of development (Negishi *et al.*, 1995). Therefore mice were generated in the lab, which allowed for tetracycline inducible expression of Zap70 protein on an endogenous Zap70-deficient background (see **Materials and Methods** section **2.1.2**). We hypothesised that administration of the tetracycline derivative doxycycline (dox) would alleviate the block at the DP stage of development, enabling the continuation of thymic development. This would allow us to characterise the kinetics of reconstitution of mature thymic compartments.

3.2 Results

3.2.1 Phenotypic characterization of TetZap70 mice

We wanted to explore the role of Zap70-dependent TCR signals beyond the DP block exhibited by *Zap70*$^{-/-}$ mice (Negishi *et al.*, 1995). Mice were generated previously in the lab to allow for tetracycline inducible Zap70 expression on an endogenous *Zap70*$^{-/-}$ background. These mice possessed a transgene encoding Zap70 and downstream internal ribosomal entry site (IRES)-Human CD2 (HuCD2) tailess reporter, under the control of a tetracycline response element (TRE) (*Zap70*^{Tre} strain hereon) (Gossen & Bujard, 1992; Saini, 2007; Saini *et al.*, 2010). These mice were crossed with a strain expressing a reverse tetracycline transactivator (rtTA) protein under control of HuCD2 expression elements (*rtTA*^{huCD2}) (Legname *et al.*, 2000; Zhumabekov *et al.*, 1995). Mice were additionally bred to an endogenous Zap70-deficient background (Negishi *et al.*, 1995), ensuring that induced *Zap70*^{Tre} transgene was the only source of Zap70 expression (generating *Zap70*^{Tre} *rtTA*^{huCD2} *Zap70*$^{-/-}$ mice, TetZap70 mice hereon) (Saini *et al.*, 2010). Zap70 induction could be achieved through the oral administration of the tetracycline derivative doxycycline (dox), in the food of these mice (refer to **Materials and Methods,** sections **2.1.2-2.1.3** and **Figure 2.1**) (Saini *et al.*, 2010).

We wanted to investigate the kinetics of thymic development beyond positive selection by inducing Zap70 for short periods of time and phenotypically characterising the first wave of developing cells. However, we first wanted to

know if the TetZap70 transgenic system enabled dox-inducible expression of Zap70 *in vivo*. To investigate this, TetZap70 mice were fed dox for ≥7 days (TetZap70^{on} hereon). Examination of the thymic T-cell phenotype by flow cytometry revealed expression of both Zap70 and the HuCD2 reporter by TetZap70^{on} thymocytes (**Figure 3.1 A**). In addition, when we compared the abundance of Zap70 in B6 and TetZap70^{on} thymocytes, we found that Zap70 was expressed at similar levels (**Figure 3.1 A,** lower panel). HuCD2 appeared to be a faithful reporter of Zap70 expression, as there was a positive correlation between Zap70 and HuCD2 expression in TetZap70^{on} thymocytes (**Figure 3.1 B**). Typically dox administration resulted in Zap70/HuCD2 expression in ~50-60% of the thymocytes (**Figure 3.1 C**). Failure to induce Zap70/HuCD2 in some thymocytes may result from variegated expression of the *Zap70^{Tre}* transgene, which lacks a locus control region (LCR) (Saini, 2007) (reviewed in (Festenstein & Kioussis, 2000)). Collectively these results indicate that dox administration to TetZap70 mice results in variegated induction of Zap70 expression *in vivo*.

Mature CD4 and CD8 SP thymocytes are absent in *Zap70$^{-/-}$* thymi due to a block in the positive selection of DP thymocytes (Negishi *et al.*, 1995). We wanted to investigate whether transgenic expression of Zap70 was sufficient to alleviate this developmental block and subsequently facilitate reconstitution of the mature SP thymic compartments. We began by asking whether SP thymic populations were present in TetZap70^{on} mice. CD4 and CD8 SP populations were indeed detectable in TetZap70^{on} thymi, in addition to B6 control mice. In

contrast, SP populations were confirmed to be absent in *Zap70*$^{-/-}$ thymi (**Figure 3.1 D,** top row). We also wanted to examine the restoration of mature T-cell compartments independently of coreceptor expression. Expression of the TCR is upregulated with maturity following positive selection (Havran *et al.*, 1987; Penit, 1990; Shortman *et al.*, 1991). CD5 is also upregulated on thymocytes that are undergoing or have completed positive selection (Azzam *et al.*, 1998) and is believed to be a negative regulator of TCR signalling (Azzam *et al.*, 2001; Tarakhovsky *et al.*, 1995). We further investigated whether tetracycline-inducible Zap70 facilitates mature thymocyte development by comparing TCR and CD5 expression on TetZap70^{on} thymocytes with B6 or *Zap70*$^{-/-}$ controls. Both TetZap70^{on} and B6 thymi showed an abundant TCR^{hi}CD5^{hi} population, whereas *Zap70*$^{-/-}$ thymocytes were uniformly TCR^{lo}CD5^{lo} (**Figure 3.1 D,** bottom row). This suggested that transgenic Zap70 expression facilitates mature thymocyte development in endogenous Zap70-deficient thymocytes. However, we did find the TCR^{hi}CD5^{hi} mature thymocyte population of TetZap70^{on} mice to be significantly reduced in frequency compared to equivalent B6 controls ($p \leq 0.001$, Student's t-test B6 n=5, TetZap70^{on} n=8) (**Figure 3.1 E**). The reduced frequency of mature cells may be partly due to transgene variegation, as the presence of Zap70-deficient cells may lead to an overrepresentation of the TCR^{lo}CD5^{lo} population. We investigated this by gating on HuCD2^{+} or HuCD2^{-} thymocytes from TetZap70^{on} mice to assess their comparative phenotypes. We found that only HuCD2^{+} thymocytes showed phenotypic restoration of mature SP thymic compartments. In contrast HuCD2^{-} cells resembled the *Zap70*$^{-/-}$ phenotype, arrested at a

$TCR^{lo}CD5^{lo}$ DP stage (**Figure 3.1 F**). Finally we asked whether transgenic Zap70 expression could restore both CD4 and CD8 lineage development equally. When we examined the frequency of CD4 and CD8 SP cells within the $TCR^{hi}CD5^{hi}$ mature thymocyte population, we found no difference in the frequency of CD4 SPs in $TetZap70^{on}$ compared to B6 mice. However there was a significant reduction in the CD8 SP frequency in $TetZap70^{on}$ mice as compared to B6 controls ($p \leq 0.05$, Students t-test B6 n=6, $TetZap70^{on}$ n=11) (**Figure 3.1 G**). Taken together, these results indicate that *Zap70*$^{-/-}$ thymocytes are blocked at the DP stage of development and are uniformly $TCR^{lo}CD5^{lo}$. However tetracycline-inducible Zap70 expression can alleviate this block in thymic development and facilitates the further maturation of dox-induced TetZap70 thymocytes.

3.2.2 Examination of the kinetics of positive selection

The temporal regulation of positive selection and subsequent CD4/CD8 lineage commitment is not well characterised. It has been suggested that CD4 SP development occurs rapidly following positive selection, whereas CD8 SPs develop with delayed kinetics (Ceredig *et al.*, 1983; Lucas *et al.*, 1993; Lundberg & Shortman, 1994). However due to technical limitations, the experimental protocols previously employed to study this have been necessarily manipulative or indirect (reviewed in (Germain, 2002) and see section **3.3 Discussion**). In contrast, the TetZap70 mice represented a system where we could synchronously restore DP arrested thymocyte development *in vivo*, with minimal experimental manipulation. We therefore asked whether TetZap70

thymocytes showed a kinetic distinction in the development of CD4 and CD8 SP lineages following Zap70 induction. To do this, adult TetZap70 mice were maintained off dox, so they resemble a *Zap70*$^{-/-}$ phenotype (**Figure 3.2 A,** left panel). We induced Zap70 expression in these mice by administering dox for 1-7 days and analysed the thymic phenotype at each timepoint. This allowed us to follow the phenotypic changes as the first wave of cells matured beyond the non-positively-selecting (non-selecting hereon) DP stage. These experiments were first performed with bone marrow (BM) chimeras, in an effort to reduce the variability in Zap70 induction observed in intact TetZap70 mice (**Figure 3.1**). BM chimeric TetZap70 mice were made, by reconstituting irradiated *Rag1*$^{-/-}$ hosts with BM from TetZap70 mice. Uninduced chimeras resembled the *Zap70*$^{-/-}$ phenotype inasmuch as they lacked both CD4 and CD8 SP populations (**Figure 3.2 A** left panel, **Figure 3.1 D**). Chimeric TetZap70 mice induced for one day also lacked CD4 and CD8 SP populations, as no CD4 SP cells were observed until at least two days after Zap70 induction. However there was a much greater delay in the developmental kinetics of CD8 SPs, as this population was not observed until at least four days after Zap70 induction (**Figure 3.2 A**). That CD4 and CD8 SPs indeed represented post-selection thymic T-cells was confirmed by first gating on the mature TCR^{hi}CD5^{hi} population (**Figure 3.2 B**). The temporal distinction between CD4 and CD8 development was further evident when we examined the CD4:CD8 ratio during the timecourse. We saw an initial increase in this ratio from approximately 15:1 on day one to approximately 80:1 on day three. The CD4:CD8 thymocyte ratio subsequently decreased between days 3-7 as the first wave of CD8 SPs

developed. By the later day 6-7 timepoints the ratio had fallen to approximately 15:1. Interestingly the CD4:CD8 ratio of thymocytes from chimeric TetZap70 mice induced for seven days was still higher than in the steady-state B6 thymus (**Figure 3.2 C**). This was consistent with the significant reduction in CD8 SPs, but normal reconstitution of CD4 SPs in TetZap70^{on} thymocytes relative to B6 mice (**Figure 3.1 G**).

We finally confirmed that the kinetics of CD4 and CD8 SP development in TetZap70 BM chimeric mice were consistent with those observed in intact TetZap70 mice, by comparing their respective frequencies of CD4 and CD8 cells after dox-induction for 1-7 days. We saw no difference in the kinetics of CD4 and CD8 SP development between chimeric or intact TetZap70 mice. In both situations we saw a rapid increase in the frequency of CD4 SPs between 0-3 days, followed by a delayed increase in the frequency of CD8 SPs between 3-5 days (**Figure 3.2 D**). The data suggested that BM chimeric mice behaved similarly to intact TetZap70 mice (**Figure 3.2**, (Saini *et al.*, 2010)) and we therefore used intact TetZap70 mice and BM chimeric mice interchangeably hereon (all subsequently referred to as TetZap70, figure legends indicate whether data are from intact or BM chimeric mice).

3.2.3 Examination of the maturation of single positive thymocytes

We next wanted to examine the continued maturation of thymocytes beyond positive selection, within the SP populations. Heat stable antigen (HSA) is expressed on DP and immature SP thymocytes, before being downregulated on

SP thymocytes as they complete their maturation (Wilson *et al.*, 1988). We therefore examined the kinetics of HSA downregulation on newly generated SP cells from TetZap70 mice induced to express Zap70 for 1-7 days. It was found that the CD4 SPs generated after 2-3 days of Zap70 induction were uniformly HSA^{hi} (**Figure 3.3 A**, middle row). We saw downregulation of HSA expression on a subset of CD4 SPs at around day 4-5. The development of the CD8 SPs by day four after Zap70 induction correlated with an almost immediate downregulation of HSA in a population CD8 SP cells (**Figure 3.3 A**, bottom row). We therefore saw that HSA was downregulated at a similar time in both CD4 and CD8 SP populations, despite the asymmetric generation of the CD4 and CD8 SP populations themselves (**Figure 3.3 A**).

We next asked how long it took newly generated SP thymocytes to emigrate from the thymus and populate peripheral lymph nodes (LN) following induction of Zap70. To do this, TetZap70 mice were induced to express Zap70 for 0-7 days and the frequency of TCR expressing cells in the LN was determined. We saw an extremely low background of TCR expressing cells between days 0-3, which may represent leaky *Zap70*^{Tre} transgene expression in a minority of cells, allowing them to overcome the block in positive selection. However there was an increase in the frequency of TCR^{+} cells between days 4-7 (**Figure 3.3 B**), which correlated with HSA downregulation in the thymus (**Figure 3.3 A**). Examining the frequency of CD4 or CD8 SP T-cells in the LN of TetZap70 mice induced for 1-7 days, revealed that CD4 and CD8 lineage T-cells both appear between days 4-7 (**Figure 3.3 C**). However, due to the initially low frequency of

peripheral T-cells, it was not clear from this analysis whether there was any temporal distinction in the thymic egress of either SP lineage.

3.2.4 Use of TCR and CD5 expression to define developmentally distinct subsets of DP thymocytes

As our data suggested that CD4 SP and CD8 SP cells developed at different times following positive selection, we wanted to identify whether phenotypic changes in positively selecting DP cells were associated with the appearance of CD4 or CD8 lineage cells respectively. TCR expression is upregulated on positively selecting thymocytes and previous studies have taken advantage of this marker to identify temporally distinct subsets of T-cells both *in vitro* and *in vivo* (Anderson *et al.*, 1994; Lucas & Germain, 1996). CD5 is a negative regulator of TCR signalling and is developmentally regulated (Azzam *et al.*, 1998; Tarakhovsky *et al.*, 1995). We therefore wished to investigate the developmental regulation of TCR and CD5 early in thymic positive selection. We first compared the TCR and CD5 expression on DP thymocytes of TetZap70 mice that had been induced to express Zap70 for different numbers of days. We found that all DP cells in uninduced mice were $TCR^{lo}CD5^{lo}$. One day after induction of Zap70, and prior to CD4 SP or CD8 SP development, we identified a population of $TCR^{intermediate}$ (TCR^{int} hereon) $CD5^{hi}$ cells, which preceded the development of the CD4 SP population at day two. Further to this, we noted the presence of $TCR^{hi}CD5^{int}$ cells by day three, which preceded the development of the CD8 SP population (**Figure 3.4 A**). We subsequently referred to these three temporally distinct DP populations in the following way:

$TCR^{lo}CD5^{lo}$ as DP1 hereon, $TCR^{int}CD5^{hi}$ as DP2 hereon and $TCR^{hi}CD5^{int}$ as DP3 hereon (**Figure 3.4 B**), in keeping with the nomenclature for temporally distinct populations of DN thymocytes (DN1-4) (Godfrey *et al.*, 1993).

To investigate the lineage relationships between these novel DP subsets and the mature SP cells, TCR and CD5 expression were analysed in the DP populations of TCR transgenic mice. F5 and OT-I T-cells express MHC-I restricted TCRs, and therefore on a *Rag1*$^{-/-}$ background will only generate CD8 lineage cells (**Figure 3.5**) (Hogquist *et al.*, 1994; Mamalaki *et al.*, 1993). A $CD4^{+}CD8^{lo}$ population was observed in OT-I *Rag1*$^{-/-}$ mice but not F5 *Rag1*$^{-/-}$ mice, which may represent cells undergoing coreceptor reversal and are thus uncommitted to either the CD4 or CD8 lineage as opposed to being MHC-I restricted CD4 SP cells (**Figure 3.5 A**) (Brugnera *et al.*, 2000). We found that all three DP populations identified in **Figure 3.4 B** were present in both the F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ TCR transgenic mice. We additionally analysed the thymic phenotype of the MHC-II restricted OT-II *Rag1*$^{-/-}$ and DO11.10 *Rag1*$^{-/-}$ TCR transgenic mice, which only generate CD4 SPs (**Figure 3.5 A**) (Barnden *et al.*, 1998; Murphy *et al.*, 1990). Intriguingly, although we saw a DP2 population in both strains, the DP3 population was absent in both cases (**Figure 3.5 B**). As a further control we examined the TCR and CD5 expression on SP populations in the transgenic mice relative to the DP2 or DP3 expression levels. In all cases, we found that the DP2 population had low surface expression of TCR relative to mature SPs. In contrast the DP3 populations in MHC-I

restricted mice had similar TCR expression compared to the mature CD8 SP population (**Figure 3.5 C**).

Next we investigated whether DP1-3 populations were present in polyclonal mice selecting only one or other of the CD4 or CD8 lineages. Mice that are homozygous null for MHC class-II genes *H2-Ab1*, *H2-Aa*, *H2-Eb1*, *H2-Eb2* and *H2-Ea* (B6.129-*H2<dlab1-Ea>*, MHC-II Δ/Δ hereon) cannot positively select CD4 SP T-cells (Madsen *et al.*, 1999), whilst mice lacking β_2-microglobulin (β_2m) (*b2m*$^{-/-}$ hereon) cannot positively select CD8 SP T-cells due to a failure of MHC-I surface expression on antigen presenting cells (APCs) (Koller *et al.*, 1990; Zijlstra *et al.*, 1990). Analysis of these strains revealed that all three DP populations were present in the MHC-II-deficient mice, whereas there was a severe reduction in the frequency of the DP3 population in the *b2m*$^{-/-}$ mice (**Figure 3.6 B**). Comparison of TCR and CD5 expression on SP populations to the DP2 and DP3 cells confirmed that the mature SP populations had higher levels of TCR expression than the DP2 population, whereas the CD8 SP population had similar levels of TCR and CD5 expression compared to the DP3 population (**Figure 3.6 C**). Taken together this data suggests that MHC-II mediated positive selection does not result in the differentiation of the DP3 population. In contrast, significant DP3 development was seen under conditions where positive selection was mediated by MHC-I.

3.2.5 DP subsets display a distinct mRNA expression profile

These data led us to hypothesise that the DP1 population consisted of cells that have yet to undergo positive selection. Furthermore they suggest that the DP2 population comprised precursors for the CD4 SP population and indirect precursors for the CD8 SP lineage, whereas the DP3 population contained direct precursors for the CD8 SP lineage, as this population was seen prior to CD8 SP development.

To investigate the proposed precursor-product relationships between DP1-3 subsets and mature SP thymocytes, we first asked whether the DP subpopulations showed differential expression of genes that are associated with either CD4 or CD8 lineage commitment. DP1-3, CD4 SP and CD8 SP cells were sorted to high purity (≥95%) from thymi of B6 mice, and gene expression of lineage development factors analysed by RT-qPCR (**Figure 3.7**). We first looked at expression of the transcription factor Gata3, which has been shown to be necessary for CD4 SP development but dispensable for CD8 SP development (Hernandez-Hoyos *et al.*, 2003; Pai *et al.*, 2003; Wang *et al.*, 2008). Levels of Gata3 in DP1 and DP3 cells were low. In contrast Gata3 expression was more highly expressed within the DP2 population (**Figure 3.7 A**, top panel). Gata3 was also highly expressed in the CD4 SP population compared to the CD8 SP population (**Figure 3.7 A**, lower panel). We next analysed the expression of Thpok, a transcription factor proposed to be a “master regulator” of CD4 SP lineage commitment (Egawa & Littman, 2008; He *et al.*, 2005; Muroi *et al.*, 2008). Thpok was absent from all DP populations

as well as CD8 SP thymocytes and was only found in the CD4 SP population (**Figure 3.7 B**). This suggests that although DP2 cells may be predisposed to selection for the CD4 lineage due to increased expression of Gata3, they are not likely to be lineage committed at this stage, as they lack Thpok expression.

Runx3 is a CD8-associated transcription factor, implicated as playing a role in *Thpok* silencing (Setoguchi *et al.*, 2008), *Cd4* repression (Taniuchi *et al.*, 2002) and necessary for CD8 SP development (Egawa *et al.*, 2007). Examining Runx3 expression revealed low levels in DP1, DP2 and CD4 SP populations. However Runx3 was selectively upregulated in DP3 thymocytes and mRNA was expressed at similar high levels in CD8 SP cells (**Figure 3.7 C**). Finally we looked at expression of Cathepsin W (encoded by the Ctsw gene), a marker used previously to identify terminally differentiated CD8 SP thymocytes (Liu & Bosselut, 2004). Ctsw was expressed at low levels in DP1-3 and CD4 SP populations and high expression was only seen in the CD8 SP population (**Figure 3.7 D**). Together these data provide evidence for the differential regulation of lineage commitment factors throughout positive selection. We hypothesised that the DP2 population may be predisposed to selection for the CD4 SP lineage since there was high expression Gata3. Similarly DP3 cells may be predisposed for CD8 SP lineage development as they express Runx3.

3.2.6 Precursor-progeny relationships between DP subsets and mature SP compartments

We wanted to directly test the hypothesised precursor-progeny relationship between the DP and SP subsets, and in addition provide confirmation of differences in the kinetics of CD4 and CD8 development. To do so, DP1-3 populations were sorted to high purity (≥90%) from congenically marked CD45.2$^+$ B6 mice and injected directly into the thymus of CD45.1$^+$ B6 hosts. This allowed us to follow the development of a wave of B6 thymocytes, with no new input to the transferred population. We first asked whether DP1 precursors differentiate into CD4 and CD8 SP thymocytes with similar kinetics as observed in TetZap70 mice. Following intrathymic injection of DP1 cells, CD4 SP cells could be identified within two days, whereas CD8 SP cells were not present until four days (**Figure 3.8 A**). This timing was indeed consistent with the developmental kinetics seen upon Zap70 induction in TetZap70 mice (**Figure 3.2 A-C**). We next asked whether DP2 cells could differentiate into either CD4 or CD8 SP cells following intrathymic transfer. DP2 precursors were found to differentiate into both CD4 and CD8 SPs. However, in this case the developmental timing was advanced by one day compared to injected DP1 cells, so that now CD4 SPs were present one day after transfer, whereas CD8 SPs were present three days after transfer (**Figure 3.8 B**). Finally we wanted to address whether the DP3 population contained precursors for both CD4 and CD8 SP lineages. In contrast to injected DP1 and DP2 populations, the DP3 precursor cells only differentiated to CD8 SPs after just 1-2 days following intrathymic transfer (**Figure 3.8 C**).

To explore whether MHC-I restricted cells necessarily transit through the DP2 population *en route* to the CD8 SP population, we examined F5 thymic development *in vivo*. In the absence of the β_2m component of MHC-I, thymocytes in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ mice are developmentally arrested at the DP stage (Koller *et al.*, 1990; Saini *et al.*, 2010) (**Figure 3.8 D**, left column). We therefore injected total F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} thymocytes into thymi of B6 CD45.1^{+} hosts (that express the MHC-I selecting ligand), thereby alleviating the block in positive selection. This allowed us to follow the developmental kinetics of a wave of F5 cells. One day after intrathymic transfer we identified F5 cells that were phenotypically DP2. In addition we saw an apparent phenotypic progression through to DP3 on days 2-3 and subsequently to the CD8 SP population on days 4-7 following intrathymic transfer (**Figure 3.8 D**). This data suggests that regardless of MHC restriction, positively selecting cells initially transit to the DP2 population after approximately one day. This DP2 population may contain the direct precursors for the CD4 SP lineage (taking one further day) but also precursors of the CD8 lineage, which additionally transit via a DP3 intermediate. This data also supports the hypothesis that the CD4 and CD8 lineages do indeed develop from phenotypically distinct populations of DPs.

3.2.7 Lineage commitment requires intrathymic signalling

To investigate whether intrathymic signals are required for the continuing development of positively selecting thymocytes, we cultured DP2 or DP3 cells overnight in medium with no additional stimuli. We found no evidence for the

phenotypic conversion of these DP2 or DP3 cells to either SP or other DP phenotypes. This suggested that following initiation of positive selection, intrathymic signals are essential for the continued maturation of both DP2 and DP3 populations (**Figure 3.8 E**).

3.2.8 Thpok mRNA is rapidly upregulated during CD4 SP development

The kinetic signalling model of T-cell development posits that positively selecting thymocytes transit through a $CD4^{hi}CD8^{lo}$ population. Such a population is proposed to be uncommitted to either the CD4 or CD8 lineage (Brugnera *et al.*, 2000) (reviewed in (Singer *et al.*, 2008)). We wanted to distinguish whether SP cells that develop 2-3 days after positive selection in TetZap70 mice represented a $CD4^{hi}CD8^{lo}$ uncommitted population or whether they included committed CD4 lineage cells. ThPOK has recently been described as being a "master regulator" of CD4 SP lineage commitment (see section **3.2.5**). We wanted to know the kinetics with which Thpok mRNA is upregulated in newly differentiated CD4 SP cells. To do this, we sorted CD4 SP cells from TetZap70 mice that had been induced to express Zap70 for two or three days. We compared Thpok mRNA expression in these cells to a variety of control populations, known to differentially express Thpok (**Figure 3.9 A**) (Muroi *et al.*, 2008). Thpok mRNA was not expressed in *Rag1*$^{-/-}$ thymocytes, in which T cell development is blocked at the DN3 stage (Godfrey *et al.*, 1993; Mombaerts *et al.*, 1992b), or in DP1 cells. We also saw no expression in CD8 SP cells. Interestingly, we found that Thpok was specifically upregulated in the newly generated CD4 SP cells of the TetZap70 mice induced to express Zap70

for two or three days, although Thpok expression was at a higher level in B6 control CD4 SPs. In contrast Thpok expression in the $CD4^{hi}CD8^{lo}$ population of MHC-II-deficient mice, was at a much lower level (**Figure 3.9 B**). This suggested that newly generated CD4 SPs in Zap70-induced TetZap70 mice rapidly upregulate Thpok expression and by implication are undergoing commitment to the CD4 lineage. Surprisingly, identically gated $CD4^{hi}CD8^{lo}$ cells from B6 mice had relatively higher levels of Thpok compared to the same population in MHC-II-deficient mice, although it was still less than observed in TetZap70 CD4 SP thymocytes that had been induced to express Zap70 for two or three days (**Figure 3.9 B**, $CD4^{hi}CD8^{lo}$ cell sorted populations are shown in **Figure 3.9 A**). This implied that MHC-II and MHC-I-restricted $CD4^{hi}CD8^{lo}$ cells exhibit distinguishing phenotypes.

3.2.9 Sensitivity to medullary chemoattractants is rapidly increased during CD4 SP development

It has been proposed that Thpok must reach a threshold of induction before lineage commitment takes place (Muroi *et al.*, 2008). Therefore it could be argued that the rapid induction of Thpok seen in newly generated CD4 SP thymocytes from TetZap70 mice fell below this threshold, as Thpok mRNA expression in newly generated TetZap70 CD4 SP thymocytes was lower than a steady state B6 CD4 SP population (**Figure 3.9 B**). We therefore wished to investigate whether the most immature CD4 SP thymocytes were lineage committed independently of Thpok expression. Concurrent with positive selection and CD4/CD8 SP lineage commitment, thymocytes migrate from the

thymic cortex to the medulla (Witt *et al.*, 2005). The upregulation of c-c motif chemokine receptor (CCR)7 on positively selecting thymocytes confers sensitivity to the medullary chemokines c-c motif chemokine ligand (CCL)19 and CCL21 and is essential for this migration (Campbell *et al.*, 1999; Misslitz *et al.*, 2004; Ueno *et al.*, 2004). We hypothesised that lineage committed CD4 SPs would express functional levels of CCR7. To test this, we first analysed CCR7 expression in steady state B6 thymocytes. CCR7 was undetectable in DP1 and DP2 thymocytes (**Figure 3.10 A**). In contrast, both immature HSA^{+} CD4 SPs and mature HSA^{-} CD4 SP expressed relatively high levels of CCR7 (**Figure 3.10 A**). We next investigated the kinetics of CCR7 upregulation in TetZap70 mice, induced to express Zap70 for two or three days. Similar to B6 mice, we saw no expression of CCR7 in either DP1 or DP2 populations of TetZap70 mice two or three days after Zap70 induction (**Figure 3.10 B**, top row). In contrast HSA^{hi} CD4 SPs generated after two or three days expressed high levels of CCR7.

These data suggested that the first CD4 SPs in TetZap70 mice rapidly upregulate sensitivity to medullary chemokines. To test whether the upregulated CCR7 was indeed functional, we analysed the capacity of TetZap70 mice to migrate towards CCL21 chemokine in a transwell chemotaxis assay. We found that neither B6 nor TetZap70 DP1 or DP2 populations showed high levels of migration. In contrast after two days of dox feeding, approximately 40% of the TetZap70 CD4 SPs were able to migrate towards CCL21 and after three days this increased to approximately 60%

(**Figure 3.10 C**). This data suggests that the rapid upregulation of CCR7 on CD4 SPs of TetZap70 mice induced to express Zap70 for two or three days was indeed functional and indicative of the final stages of SP maturation.

3.2.10 CD4 SP and CD8 SP thymocytes migrate from the thymic cortex to the medulla with different kinetics

We next sought to determine whether the rapidly upregulated CCR7 expression seen in CD4 SPs from TetZap70 mice was indeed functional *in vivo*. We asked how long it took for TetZap70 CD4 and CD8 SP cells to migrate from the thymic cortex to medulla following restoration of positive selection by Zap70 induction. TetZap70 mice were induced to express Zap70 for different numbers of days and thymic sections were subsequently analysed by confocal microscopy. In dox-free mice (d0), medullary areas were devoid of T-cells and reduced in size, in keeping with the phenotype of *Zap70*$^{-/-}$ mice (**Figure 3.11**, d0) (Negishi *et al.*, 1995). In contrast, two days after Zap70 induction, we saw CD4 SPs had migrated to thymic medulla that was concomitant to an enlargement of the medulla (**Figure 3.11**, d2). There was an apparently increased density of CD4 SPs in the thymic medulla thee days after Zap70 was induced in the TetZap70 mice, whereas CD8 SPs were absent at this timepoint (**Figure 3.11**, d3). CD8 SPs were subsequently found in the thymic medulla of TetZap70 mice that had been induced to express Zap70 for four or more days, fitting with the detection of CD8 SPs by flow cytometry at these later timepoints (**Figure 3.2 A-C, Figure 3.11**). Collectively this data suggests that CD4 and CD8 SP thymocytes

migrate to the thymic medulla with different kinetics following Zap70 induction in TetZap70 mice.

3.3 Discussion

In this chapter we have investigated the temporal kinetics and phenotypic progression of SP thymocyte development following positive selection. To do so we used a tetracycline-inducible Zap70 mouse as a tool, enabling us to experimentally induce Zap70 expression in otherwise Zap70-deficient thymocytes. In the absence of Zap70 thymocytes are arrested in the DP stage of development, however experimental induction of Zap70 allowed us to alleviate this block. This permitted us to induce Zap70 for 1-7 days in TetZap70 mice and follow the development of the first wave of positively selecting cells. We found that lineage committed CD4 SP and CD8 SP populations developed with temporal distinction. The CD4 SP cells rapidly developed as they induced Thpok and migrated from the thymic cortex to medulla after only two days following positive selection. In contrast lineage committed CD8 SPs were only present four days after positive selection as they were only found in the medulla at this later time. Furthermore we identified temporally distinct DP populations (termed DP1-3) that possess different developmental potential. The DP1 population consisted of non-selecting cells. In contrast the DP2 population contained the precursors for CD4 and CD8 lineages, whereas the DP3 population only contained precursors for the CD8 SP lineage. In addition all of these DP populations required intrathymic signals to complete their maturation,

suggesting that none of the DP cells were lineage committed. Importantly, analysis of DP1-3 populations permitted study of temporally distinct subsets of positively selecting thymocytes, a distinct advantage over using coreceptor expression levels alone. We also investigated the kinetics of thymic emigration of mature cells. Our data suggested that T-cells reside in the thymic medulla for a matter of days before emmigrating to the periphery 4-5 days after positive selection.

The TetZap70 mice allowed us to restore thymic development beyond the DP stage with minimal experimental manipulation, in order to examine the kinetics of subsequent thymic development. Such a system has advantages in that it does not require *ex vivo* manipulation of thymocytes. In addition, Zap70 induction occurred extremely rapidly following dox administration (Saini *et al.*, 2010) and Zap70 itself was expressed at physiological levels (**Figure 3.1 A**). This contrasts with the tetracycline inducible Lck transgenic mice, which express 10-fold higher Lck in thymocytes relative to wild-type (WT) (Legname *et al.*, 2000). Furthermore, most defects in TCR signalling machinery result in a block at the β-selection developmental checkpoint (reviewed in (Germain, 2002)). In contrast Zap70 exhibits an almost complete redundancy with the related kinase Syk in the DN3 stage, allowing for TCR signal transduction during β-selection (Chan *et al.*, 1994b; Kadlecek *et al.*, 1998; Palacios & Weiss, 2007). Therefore Zap70-deficient thymocytes are unusual, as they exhibit a later block in development at the DP stage, where they are poised prior to positive selection. Induction of Zap70 expression in DP arrested thymocytes of

TetZap70 mice represents an excellent model to investigate the kinetics of T-cell development beyond the DP stage.

We subsequently used the TetZap70 mice to probe the temporal kinetics with which SP thymocyte lineages develop. Following restoration of Zap70, CD4 SP cells developed early, by day two, whereas we saw no CD8 SP development until at least four days following positive selection (**Figure 3.2 A-C**). This is consistent with data suggesting that fetal CD4 SP and CD8 SP thymocyte populations develop with temporally distinct kinetics during thymic ontogeny (Ceredig *et al.*, 1983). We have additionally shown this to be the case in the adult thymus. Our data agrees somewhat with that of researchers who labelled thymocytes with a 5-Bromodeoxyuridine (BrdU) and followed their subsequent phenotypic transition (Baron & Penit, 1990; Lucas *et al.*, 1993; Lucas *et al.*, 1994), who also found that CD4 SPs develop earlier than CD8 SPs, although there are discrepancies in the precise timings of these events between these studies and our own. Whereas we saw development of CD4 and CD8 SPs two and four days after positive selection respectively, two studies by Lucas et al report more delayed kinetics suggesting that CD4 SPs develop between 3-6 days, whereas CD8 SPs developed between 5-8 days following BrdU labelling (Lucas *et al.*, 1993; Lucas *et al.*, 1994). An explanation could be that BrdU labels primarily cycling DN3-DN4 thymocytes (Baron & Penit, 1990; Penit *et al.*, 1995), so this delay in CD4 and CD8 SP developmental kinetics may incorporate the additional thymic maturation before cells reach the DP stage. The precise timings of CD4 and CD8 SP development in Zap70-induced

TetZap70 thymocytes more closely resembles an *in vitro* reaggregate thymic organ culture (RTOC) system where CD4 SPs were found by day 2-3 and CD8 SPs by day 3-4 (Anderson *et al.*, 1994). Indeed our findings suggest that such an *in vitro* system is physiologically relevant with regards to the developmental kinetics of positive selection.

A possible criticism of the TetZap70 system is that the kinetics of positive selection in TetZap70 mice may be an artefact of transgenic Zap70 expression, which does not precisely match the endogenous expression of WT mice. However, we were able to verify these timings of lineage commitment by examining the development of B6 DP1 thymocytes following their intrathymic transfer to congenically marked B6 hosts. The kinetics of development precisely mirrored that of TetZap70 mice: CD4 SPs developed two days following injection of a non-selecting (DP1) population, whereas CD8 SPs were only seen four days after transfer (**Figure 3.2 A-C**, **Figure 3.8 A**). Not only does this suggest that the kinetics of positive selection in TetZap70 mice are representative of a WT situation, but this mutually supports the use of intrathymic transfers to study the kinetics of CD4 and CD8 positive selection. Indeed Lundberg et al. report almost identical developmental timings with regards to CD4 and CD8 development following transfer of $CD3^{lo}$ DP cells to WT hosts (Lundberg & Shortman, 1994). Nevertheless, a limitation of the intrathymic transfer technique is that poor cell recovery precludes more detailed analysis of cells. The TetZap70 system allowed us to analyse smaller

populations of cells, such as the DP2 and DP3 populations in considerable detail (compare TCR and CD5 expression profiles in **Figure 3.4** and **Figure 3.8**)

In our study we examined not only the coreceptor phenotype during CD4 and CD8 development, but also whether these SP cells were indeed lineage committed. We found that temporally distinct development of phenotypically CD4 and CD8 SP cells was also accompanied by temporal distinction with regards to lineage commitment. CD4 SPs found in TetZap70 mice that had been induced to express Zap70 for two or three days expressed high levels mRNA for the "master regulator" of CD4 lineage commitment Thpok (He *et al.*, 2005) (**Figure 3.9 B**), which has been shown previously to correlate well with ThPOK protein expression (Sun *et al.*, 2005). This suggested that some of the CD4 SPs generated after two days were indeed lineage committed. However, recent studies suggest that ThPOK must reach a threshold to induce CD4 lineage specification (Muroi *et al.*, 2008). To refute the argument that the newly generated CD4 SPs may express ThPOK at a level below the lineage commitment threshold, it was important to confirm the lineage commitment of CD4 SP cells in another way. We did so by examining the migration of the SP cells from the thymic cortex to medulla, which occurs following positive selection (reviewed in (van Ewijk, 1991)). CD4 SP cells were shown to rapidly upregulate CCR7 (**Figure 3.10 A-B**), increase sensitivity to medullary derived chemokines (**Figure 3.10 C**) and were found to reside within the thymic medulla within two days of Zap70 restoration (**Figure 3.11**). This led us to conclude that whereas some of the CD4 SP cells are indeed CD4 lineage committed by day two, the

CD8 SPs were unlikely to be lineage committed until four days after positive selection (**Figure 3.2 A-C**, **Figure 3.8 A**).

It could be argued that the DP3 population represents committed CD8 lineage cells, as they only contained precursors for the CD8 SP lineage (**Figure 3.8 C**) and expressed similar levels of the CD8 lineage associated factor Runx3 (**Figure 3.7 C**). Indeed Runx3 was expressed with a similar abundance in DP3s relative to CD8 SPs, fitting with its direct implication in CD4 silencing in CD8 SP-committing cells (Taniuchi *et al.*, 2002). However, the DP3 cells did not spontaneously downregulate the CD4 coreceptor to phenotypically resemble CD8 SPs following overnight culture in medium alone (**Figure 3.7 E**). That they do so one day after intrathymic transfer to B6 host mice suggests that intrathymic signals are required to complete DP3 maturation (**Figure 3.7 C**). Furthermore the DP3 population does not express high mRNA levels of the CD8 lineage terminal differentiation marker Ctsw (**Figure 3.8 E**, **Figure 3.7 D**) (Liu & Bosselut, 2004). In support of the hypothesis that DP3 cells are not lineage committed, it has recently been shown that Runx3 overexpression in cultured DP thymocytes is not sufficient to confer CD8 lineage commitment (Park *et al.*, 2010). Furthermore, the requirement for additional intrathymic signals in CD8 precursor cells is fitting with the concept of a “proof-reading” mechanism during CD8 lineage commitment (Liu *et al.*, 2003). Whether the hypothesised intrathymic signal required for DP3 cells to commit to the CD8 lineage is mediated by the TCR signalling, cytokine signalling, or some other factor remains to be determined. Nevertheless we suggest these previous studies are

compatible with our conclusion that the CD8 SPs are not lineage committed until four days after initiating positive selection. However, the CD8 lineage choice may be predetermined at the DP3 stage, as this population contains precursors for the CD8 SP and not the CD4 SP lineage (**Figure 3.8 C**).

The conclusion that CD4 and CD8 lineage commitment occurs with kinetic distinction is in direct opposition to previous models of positive selection. Symmetric lineage commitment models suggest that CD4 and CD8 commitment occurs with identical kinetics, but that residual levels of CD4 coreceptor remain on the cell surface, making such cells appear to be DP (Barthlott *et al.*, 1997; Lucas & Germain, 1996). However, advances in understanding the genetic control of CD4 versus CD8 development have enabled us to investigate the lineage potential of selecting thymocytes in greater detail. We additionally hypothesise that the early differentiation of CD4 SPs relative to CD8 SPs may enable the CD4 lineage to be permissive for further thymic differentiation, for example of Treg cells (Lio & Hsieh, 2008) (reviewed in (Josefowicz & Rudensky, 2009)).

A major obstacle in studying positive selection is the lack of markers allowing researchers to phenotypically and kinetically distinguish developmental intermediate populations of cells. Many previous studies have employed tight gating on populations based on differences in CD4 or CD8 coreceptor expression (reviewed in (Germain, 2002) and (Kappes & He, 2005)). It has been argued that modulations of coreceptor expression have functional implications on positive selection. For example, the initial downregulation of the

CD8 coreceptor (making cells phenotypically $CD4^{hi}CD8^{lo}$) allows cells to discriminate an MHC-I versus MHC-II mediated positive selection signals that are otherwise thought to be identical (Brugnera *et al.*, 2000; Singer *et al.*, 2008). Contrary to this view, however, we found that Thpok was expressed at much reduced levels in the $CD4^{hi}CD8^{lo}$ population of MHC-II-deficient mice as compared to B6 controls (**Figure 3.9 B**). This suggests that the WT $CD4^{hi}CD8^{lo}$ population may be heterogeneous with regards to gene expression and stage of selection. Furthermore when we examined CD4 and CD8 coreceptor expression on F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/--}$ TCR transgenic thymocytes, we found that the $CD4^{hi}CD8^{lo}$ population was infact absent in F5 *Rag1*$^{-/-}$ thymi but intact in OT-I *Rag1*$^{-/-}$ thymi, suggesting it is not an obligate stage during CD8 SP development (**Figure 3.5**). The $CD4^{hi}CD8^{lo}$ population has been suggested to correlate with increasing avidity for spMHC and thus positive selection signal strength (Ohashi *et al.*, 1993; Singer *et al.*, 2008) (reviewed in (Hogquist, 2001)). This is in agreement with our data, as OT-I *Rag1*$^{-/-}$ T-cells have a higher avidity for spMHC than F5 *Rag1*$^{-/-}$ mice (Ge *et al.*, 2004) (and see **Chapter 5**). We therefore conclude that our findings support a growing body of evidence to suggest that the intermediate stages of positive selection cannot be robustly distinguished based on coreceptor expression alone.

The suggestion that the $CD4^{hi}CD8^{lo}$ population consists of cells that are phenotypically diverse further highlights the need for additional phenotypic markers that differentiate the intermediate stages of positive selection. We described a method in this chapter that involved analysing TCR and CD5

expression on broadly gated DP cells. We have termed three newly identified DP subsets DP1-3 (**Figure 3.3-3.6, Figure 3.8**). Our studies lead us to suggest a new model for the phenotypic progression occurring during CD4 and CD8 lineage commitment. We suggest that all cells undergoing positive selection are initially recruited to the DP2 population. The DP2 population contains the direct precursors of CD4 SP and indirect precursors of CD8 SP lineages. We hypothesise that MHC-I restricted thymocytes selecting to the CD8 SP lineage must transit from the DP2 to CD8 SP population via the DP3 population (**Figure 3.12**). Critically, analysis of DP1-3 populations permits the identification of thymocyte populations in temporally distinct intermediate stages of positive selection, in a manner not possible using other phenotypic markers such as CD4 and CD8. We therefore hypothesise that analysis of the DP1-3 populations will give new insight into the regulation of the CD4/CD8 lineage decision.

For example, analysis of gene expression within the DP1-3 populations has allowed us to gain new insight into the kinetic regulation of transcription factors involved in the CD4/CD8 lineage decision. Gata3 is specifically upregulated in the DP2 population, suggesting these cells may be predisposed to selection for the CD4 SP lineage (**Figure 3.7 A**). Gata3 has been shown to be indispensible for CD4 SP development. In addition, it has been suggested that Gata3 is directly regulated by TCR signalling in positively selecting DPs (Hernandez-Hoyos *et al.*, 2003; Maurice *et al.*, 2007). Furthermore Gata3 is proposed to be directly upstream of Thpok (Wang *et al.*, 2008). Our data entirely support these

findings, as the DP2 population was found to contain the direct precursors for the CD4 SP population (**Figure 3.8 B**) and had upregulated expression of Gata3 compared to non-selecting cells (**Figure 3.7 A**), whereas Thpok mRNA was not expressed until the CD4 SP stage (**Figure 3.7 B**). Future studies will investigate whether Gata3 is differentially regulated in the DP2 population of MHC-I- or MHC-II-deficient mice. In addition microarray analysis of the DP populations in MHC-I- and MHC-II-deficient mice may enable the identification of novel factors involved in positive selection that are induced specifically by MHC-I or MHC-II-mediated selection signals.

We have also addressed the thymic maturation events that occur following positive selection and SP development. We found that after restoration of Zap70 in TetZap70 mice, CD4 SPs were found to reside in the thymic medulla after two days, whereas CD8 SPs were not found in the medulla until four days after Zap70 was induced. Concomitantly with this, we saw an apparent enlargement of the thymic medulla (**Figure 3.11**, compare d0 with d2). This data is in agreement with implications for a role of SP T-cells in medullary maturation (Shores *et al.*, 1991; Surh *et al.*, 1992). Indeed the TetZap70 system may provide a useful tool to investigate medullary thymic epithelial cell (mTEC) development in an adult thymus. It has been described that mTEC maturation depends on interactions of CD4 SP cells, which may additionally include lymphoid tissue inducer (LTi) cells (reviewed in (Anderson *et al.*, 2007)). Restoration of thymic development in TetZap70 mice may enable future studies to dissect the relative role of $\alpha\beta$-lineage T-cells to this maturation process. In

addition, analysis of thymic restoration in MHC-II or MHC-I-deficient TetZap70 mice may give insights as to whether the CD4 and CD8 SPs can contribute equivalently to mTEC maturation and proliferation.

Our studies additionally gave insight to the question of how long a T-cell must reside in the thymic medulla following CD4/CD8 lineage commitment. Data presented in this chapter show the potential for a rapid emigration of thymocytes from the thymic medulla, which we first identify on day four (**Figure 3.3 B**). This agrees with some researchers (McCaughtry *et al.*, 2007; Tough & Sprent, 1994), although contrasts with a study employing semi-continuous [^{3}H]-thymidine labelling of total thymocytes, that only identified an accumulation of peripheral T-cells after ~20 days (Egerton *et al.*, 1990). It has been hypothesised that thymic emigration is a stochastic process, potentially allowing for T-cells to have a wide distribution of medullary residency times and referred to as the "lucky-dip hypothesis" (Kelly & Scollay, 1990) (reviewed in (Scollay & Godfrey, 1995)). Such a model cannot be ruled out by our analysis of thymic egress in Zap70 induced TetZap70 mice.

We find evidence to suggest that CD4 SPs may be subject to medullary negative selection for a longer period than CD8 SPs. We see CD4 SP cells resident in the thymic medulla by one day after Zap70 induction, whereas CD8 SP cells do not appear in the thymic medulla until day four (**Figure 3.11**). Furthermore we found that TCR^{+} cells egress from the thymus at around 4-5 days following positive selection in TetZap70 mice (**Figure 3.3 C**). Although our experiments could not pinpoint whether there was an asymmetric egress of CD4

or CD8 lineage cells, evidence from recent studies suggests that this is not the case. Two groups have tracked recent thymic emigrants (RTEs) by analysing the decay in GFP abundance in mice where GFP expression was controlled by the *Rag2* promotor. GFP transcription is turned off at the DP stage, but due to its long half-life, GFP protein expression persists as it is slowly turned over at a constant rate. Therefore, the constant reduction in GFP expression allows it to be used as a molecular clock (Boursalian *et al.*, 2004). Using this method, it was found that CD4 and CD8 cells exported to the periphery had identical levels of GFP, taken to suggest that cells migrate at the same developmental stage from the medulla (McCaughtry *et al.*, 2007). When taken with our data suggesting that CD4 SPs migrate to the thymic medulla earlier than CD8 SPs (**Figure 3.11**), this suggests that CD8 SPs may have a shorter medullary residency time, relative to CD4s. Such a result implies that CD8 SPs may be less susceptible to negative selection in the medulla relative to CD4 SPs. Whether this has implications on autoimmunity remains to be investigated. Typically CD8 T-cell activation requires CD4 help (reviewed in (Wong & Pamer, 2003)), so a more stringent negative selection of autoreactive CD4 SP cells may indirectly prevent CD8-mediated autoimmunity. Perhaps slowing the thymic egress of CD8 SPs to enable more efficient negative selection may have therapeutic benefits in cases where CD4 tolerance is broken.

In conclusion the results presented in this chapter have described three novel DP subpopulations with different differentiation potential. The DP2 population could give rise to both CD4 SPs and CD8 SPs, whereas the DP3 population

could give rise to CD8 SPs alone. This leads us to suggest a model by which CD4 and CD8 lineage development occurs with temporal distinction, due to the underlying temporal differences in the direct precursor populations (**Figure 3.12**). As MHC-I signalled cells transit through the DP2 population without receiving a CD4 lineage commitment signal, we hypothesised that differential signalling in the DP2 population may direct the CD4 versus CD8 fate decision. We therefore investigated the signalling requirements for positive selection and lineage commitment in **Chapter 4**.

Figure 3.1 Tetracycline inducible Zap70 expression promotes reconstitution of mature thymic development

TetZap70 mice were fed dox for ≥7 days (TetZap70^{on}, red line) and their thymocytes were analysed by flow cytometry, along with B6 (black line) and *Zap70*$^{-/-}$ (grey fill) controls. (**A**) Histograms show HuCD2 and Zap70 expression on live thymocytes. (**B**) Density plot shows co-staining of Zap70 and HuCD2 on live TetZap70^{on} thymocytes. (**C**) Box and whisker plots show the frequency of HuCD2^{+} thymocytes (n=8) or Zap70^{+} thymocytes (n=4) in live gated TetZap70^{on} thymocytes. (**D**) Density plots show expression of CD4 against CD8 and CD5 against TCR on live thymocytes from B6, *Zap70*$^{-/-}$ and TetZap70^{on} mice. (**E**) Box plots show TCR^{hi}CD5^{hi} cells as a frequency of total live thymocytes for B6 (n=5), TetZap70 (n=9) and *Zap70*$^{-/-}$ (n=5) mice. *** p≤0.001 (Student's t-test). (**F**) Histogram shows HuCD2 expression on live TetZap70^{on} thymocytes in addition to HuCD2^{+} and HuCD2^{-} gates. Density plots show CD4 against CD8 expression and CD5 against TCR expression for HuCD2^{+} or HuCD2^{-} populations as indicated. (**G**) Box plots show frequencies of CD4 SP and CD8 SP populations expressed as a percentage of the TCR^{hi}CD5^{hi} mature thymocyte population, from B6 (n=6) and TetZap70^{on} (n=11) mice. * p≤0.05 (Student's t-test). Data are representative of three or more independent experiments.

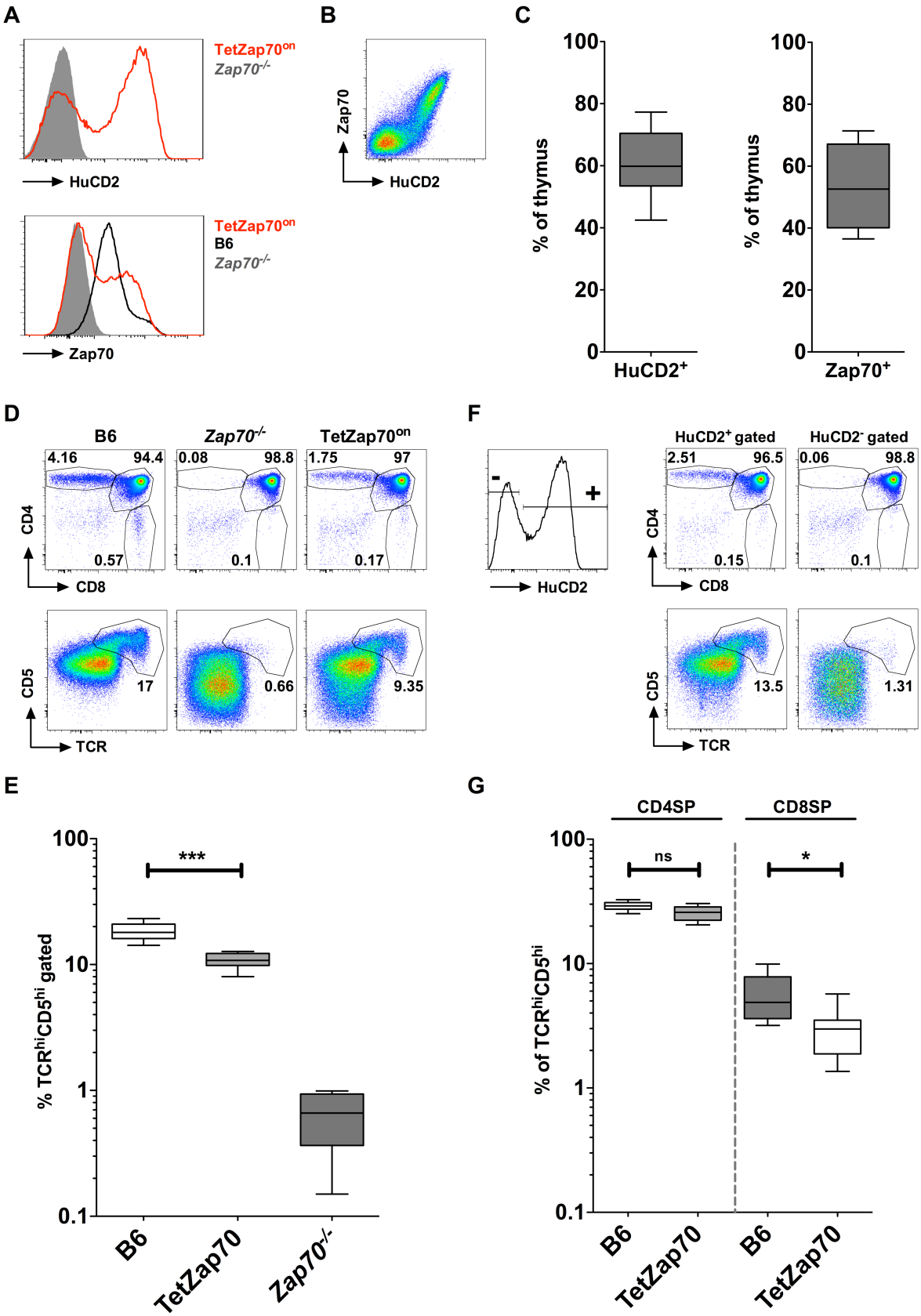

A
TetZap70on
Zap70-/-
HuCD2
TetZap70on
B6
Zap70-/-
Zap70
B
Zap70
HuCD2
C
% of thymus
HuCD2+
Zap70+
D
B6
Zap70-/-
TetZap70on
CD4
CD8
CD5
TCR
F
HuCD2
HuCD2+ gated
HuCD2- gated
E
% TCRhiCD5hi gated
B6
TetZap70
Zap70-/-
G
CD4SP
CD8SP
ns
*
% of TCRhiCD5hi
B6
TetZap70

Figure 3.2 Mature CD4 SP and CD8 SP thymocytes develop with distinct kinetics following positive selection in TetZap70 mice.

BM chimeras were made by reconstituting irradiated *Rag1*$^{-/-}$ mice with TetZap70 BM. TetZap70 BM chimeras or intact TetZap70 mice were fed dox for 0-8 days. B6 mice were also analysed as controls. Expression of CD4, CD8, TCR, CD5 and HuCD2 was analysed by flow cytometry. (**A**) Density plots show CD4 against CD8 on live thymocytes (d0 TetZap70 chimeras or B6 controls) or HuCD2^{+} TetZap70 chimeric thymocytes (d1-7). (**B**) Density plots show CD4 against CD8 on TCR^{hi}CD5^{hi} gated live thymocytes. Data in (**A**-**B**) are representative of ≥3 independent experiments. (**C**) The line graph shows the mean CD4:CD8 SP ratio in TCR^{hi}CD5^{hi} gated thymocytes from intact TetZap70 mice or B6 controls. Each point represents ≥2 replicate mice, error bars represent sd. (**D**) Line graph shows the frequency of TCR^{+} CD4 SP (circles) and TCR^{+} CD8 SP thymocytes (squares) as a percentage of total cells (d0), or HuCD2^{+} induced cells (d1-8). Separate data sets are shown for chimeric TetZap70 (red lines) and intact TetZap70 mice (blue lines). Each timepoint represents ≥2 replicate mice. Data collection and analysis for intact TetZap70 mice in part (**D**) was performed by Moni Saini and Ben Seddon.

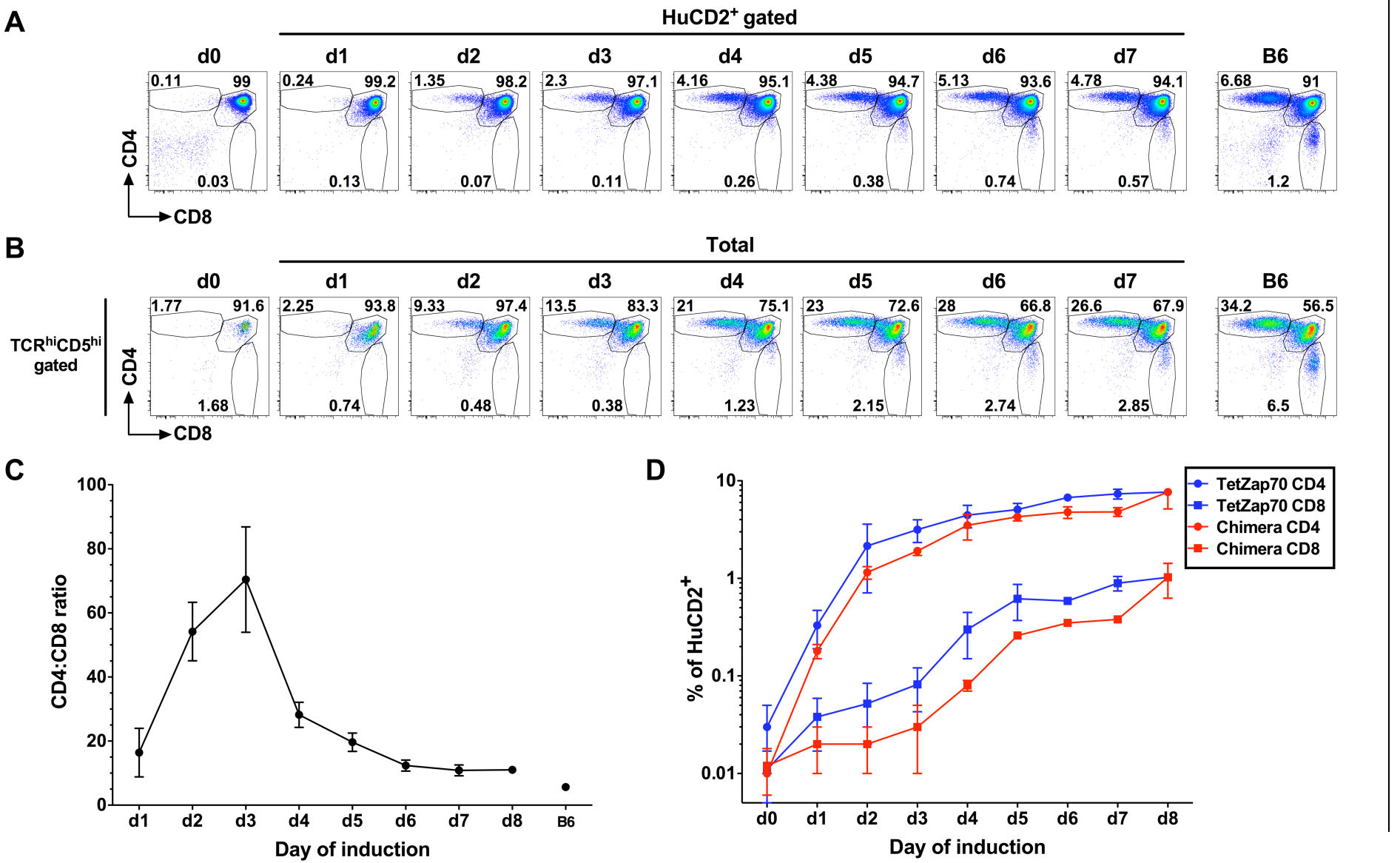

A
HuCD2+ gated
d0
d1
d2
d3
d4
d5
d6
d7
B6
0.11
99
0.03
0.24
99.2
0.13
1.35
98.2
0.07
2.3
97.1
0.11
4.16
95.1
0.26
4.38
94.7
0.38
5.13
93.6
0.74
4.78
94.1
0.57
6.68
91
1.2
CD4
CD8
B
Total
TCRhiCD5hi gated
1.77
91.6
1.68
2.25
93.8
0.74
9.33
97.4
0.48
13.5
83.3
0.38
21
75.1
1.23
23
72.6
2.15
28
66.8
2.74
26.6
67.9
2.85
34.2
56.5
6.5
C
CD4:CD8 ratio
Day of induction
D
% of HuCD2+
Day of induction
TetZap70 CD4
TetZap70 CD8
Chimera CD4
Chimera CD8

Figure 3.3 Maturation and thymic egress of TetZap70 T-cells

BM chimeras were made by reconstituting irradiated *Rag1*$^{-/-}$ mice with TetZap70 BM. TetZap70 chimeras or intact TetZap70 mice were left off dox or fed dox for 1-7 days (indicated as d1-7). Expression of CD4, CD8, TCR, CD5 and HSA was analysed by flow cytometry. (**A**) Density plots show analysis for TetZap70 chimeric mice induced for 1-7 days and B6 controls. Density plots on the top row show CD4 against CD8 expression on TCR^{hi}CD5^{hi} gated thymocytes. Density plots on the middle row show HSA against TCR, for CD4 SP thymocytes (CD4 SP gating is shown on top row plots). Density plots on the bottom row show HSA against TCR, for CD8 SP thymocytes (CD8 SP gating is shown on the top row plots). Data is representative of three independent experiments. (**B**) Line graph shows TCR^{+} cells as a frequency of live lymphocytes, in the LNs of intact TetZap70 mice. (**C**) Line graph shows TCR^{+} CD4 SPs (red line) or TCR^{+} CD8 SPs (blue line) as a frequency of lymphocytes, in the LNs of intact TetZap70 mice.

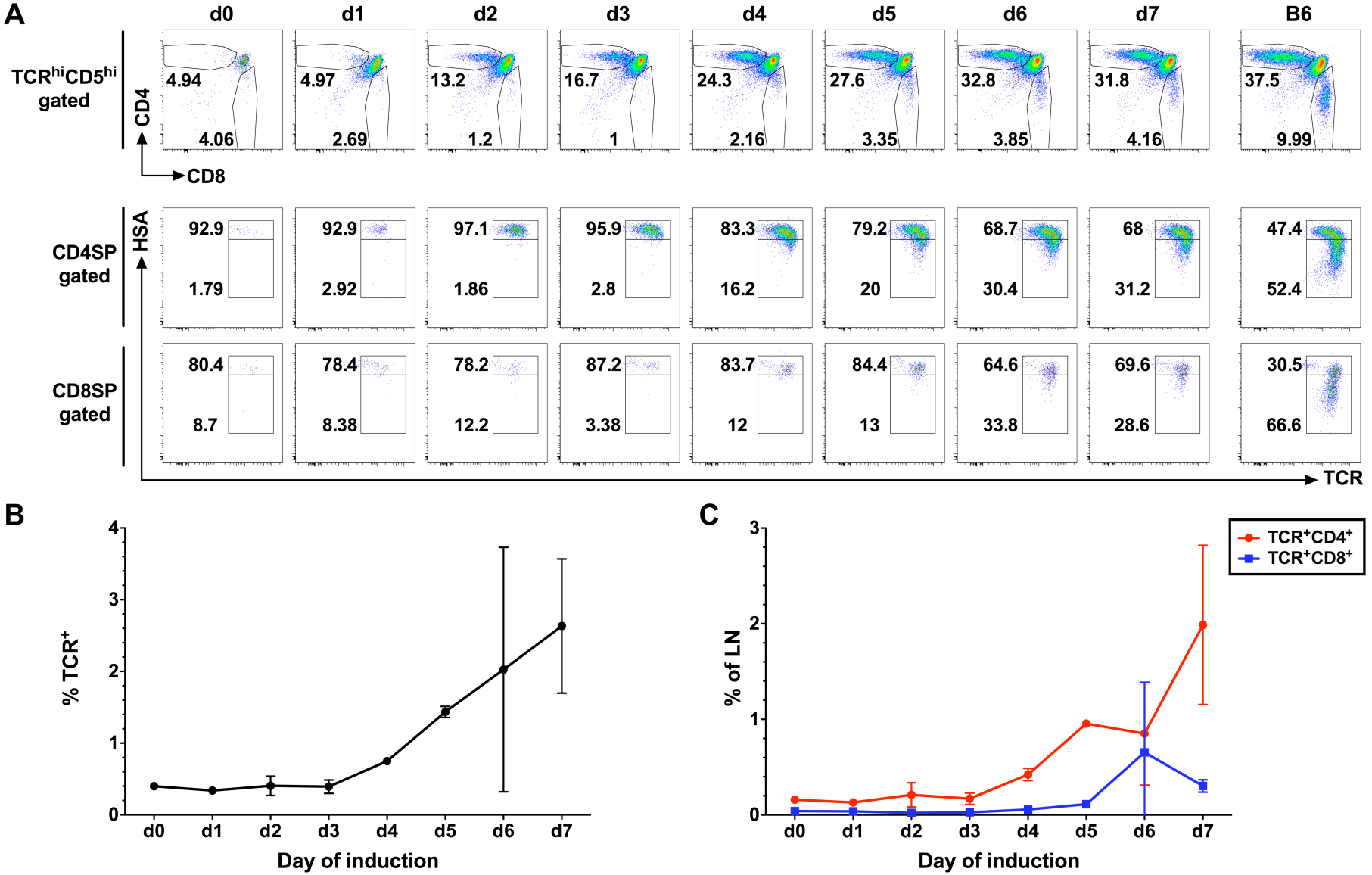

A
d0
d1
d2
d3
d4
d5
d6
d7
B6
TCRhiCD5hi gated
CD4
CD8
4.94
4.06
4.97
2.69
13.2
1.2
16.7
1
24.3
2.16
27.6
3.35
32.8
3.85
31.8
4.16
37.5
9.99
CD4SP gated
HSA
92.9
1.79
92.9
2.92
97.1
1.86
95.9
2.8
83.3
16.2
79.2
20
68.7
30.4
68
31.2
47.4
52.4
CD8SP gated
80.4
8.7
78.4
8.38
78.2
12.2
87.2
3.38
83.7
12
84.4
13
64.6
33.8
69.6
28.6
30.5
66.6
TCR
B
% TCR+
Day of induction
C
% of LN
TCR+CD4+
TCR+CD8+
Day of induction

Figure 3.4 Temporally distinct DP populations are identified by differential TCR and CD5 expression

(**A**) BM chimeras were made by reconstituting irradiated *Rag1*$^{-/-}$ mice with TetZap70 BM. TetZap70 chimeras were left off dox or were fed dox for 1-7 days (indicated as d1-7). Expression of CD4, CD8, TCR, CD5 and HuCD2 was analysed by flow cytometry. Density plots on the top row show CD4 against CD8 on live thymocytes (d0 TetZap70 chimeras or B6 controls) or HuCD2^{+} TetZap70 thymocytes (d1-7). The top row plots are duplicated from **Figure 3.2 A** to show DP gating. Density plots on the bottom row show CD5 against TCR for DP thymocytes. (**B**) Enlarged density plot shows CD5 against TCR for DP gated B6 thymocytes. DP1 (TCR^{lo}CD5^{lo}), DP2 (TCR^{int}CD5^{hi}) and DP3 (TCR^{hi}CD5^{int}) populations are indicated on the plot. Results in (**A-B**) are representative of ≥3 independent experiments.

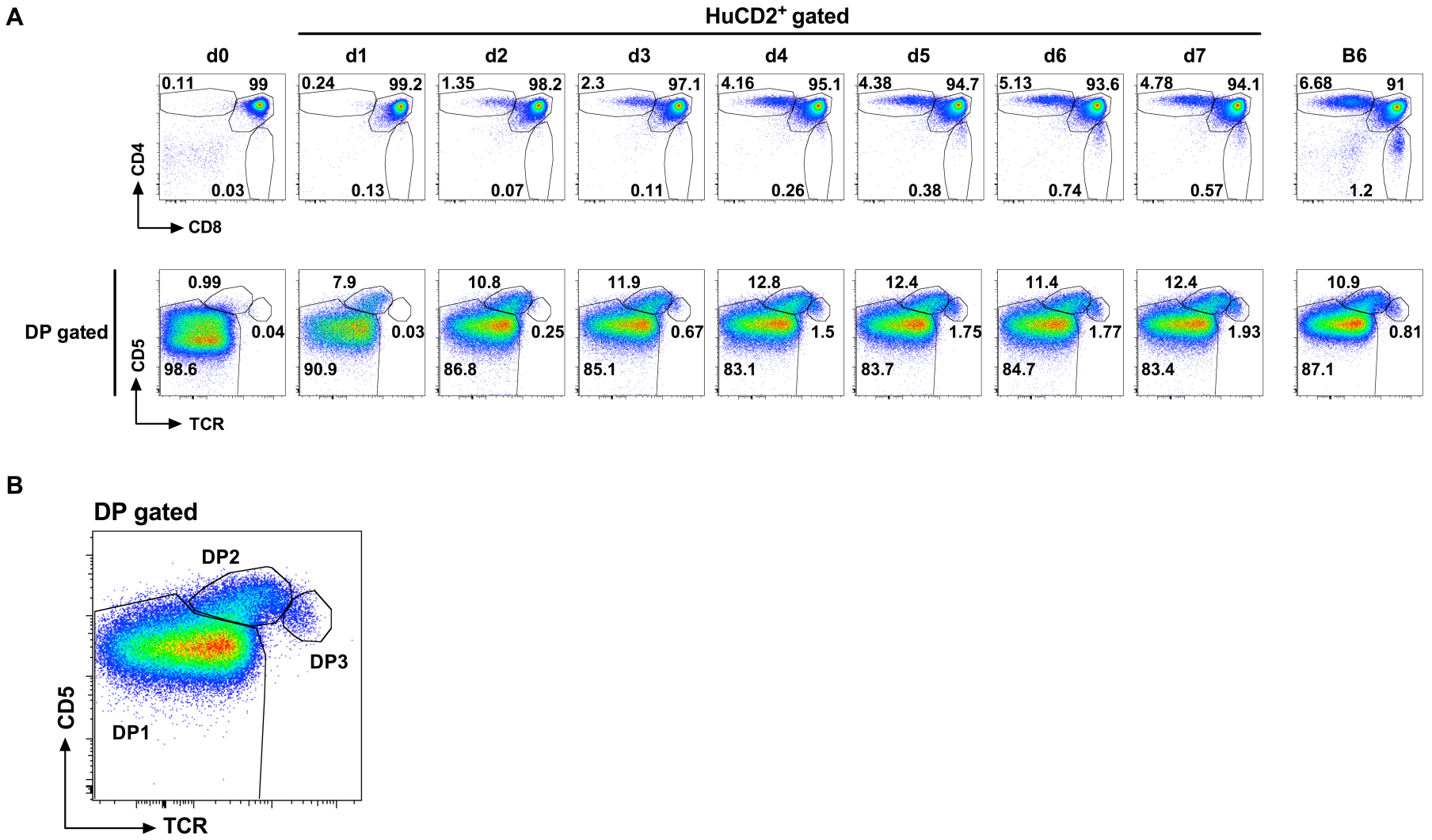

A
HuCD2+ gated
d0
d1
d2
d3
d4
d5
d6
d7
B6
CD4
CD8
0.11
99
0.03
0.24
99.2
0.13
1.35
98.2
0.07
2.3
97.1
0.11
4.16
95.1
0.26
4.38
94.7
0.38
5.13
93.6
0.74
4.78
94.1
0.57
6.68
91
1.2
DP gated
CD5
TCR
0.99
0.04
98.6
7.9
0.03
90.9
10.8
0.25
86.8
11.9
0.67
85.1
12.8
1.5
83.1
12.4
1.75
83.7
11.4
1.77
84.7
12.4
1.93
83.4
10.9
0.81
87.1
B
DP gated
DP2
DP3
DP1
CD5
TCR

Figure 3.5 Analysis of the thymic phenotype of TCR transgenic mice

MHC-I restricted F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice, MHC-II restricted OT-II *Rag1*$^{-/-}$ and DO11.10 *Rag1*$^{-/-}$ mice and B6 or BALB/c control mice, were analysed by flow cytometry, for the expression of CD4, CD8, TCR and CD5. (**A**) Density plots show CD4 against CD8 expression for live thymocytes from indicated strains of mice. CD4 SP, CD8 SP and DP gates in transgenic mice are based on a WT control. (**B**) Density plots show CD5 against TCR expression on DP gated thymocytes. (**C**) Density plots show CD5 against TCR expression for CD4 SP and/or CD8 SP populations. Density plots also show DP2 and DP3 gates from (**B**). Data in (**A-C**) is representative of ≥3 replicate mice.

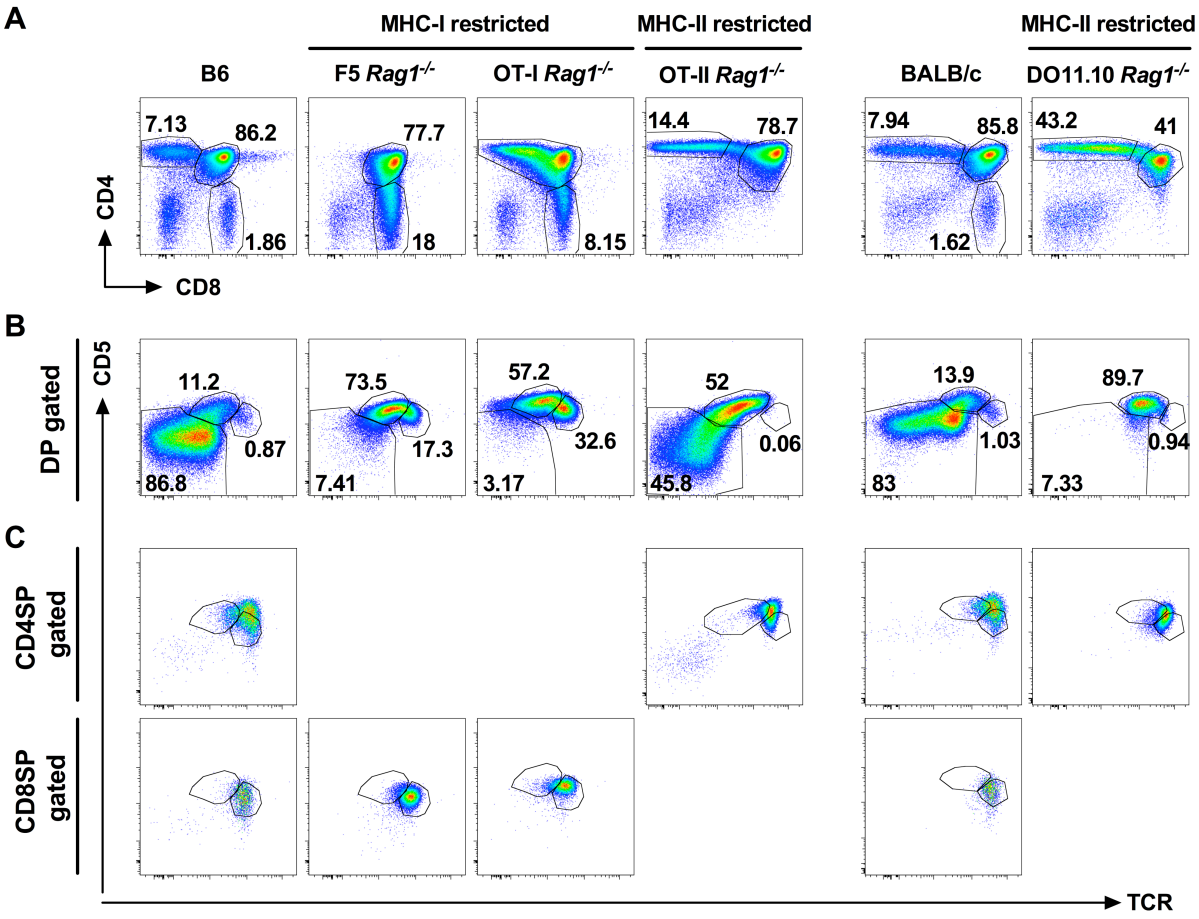

A
MHC-I restricted
MHC-II restricted
MHC-II restricted
B6
F5 Rag1-/-
OT-I Rag1-/-
OT-II Rag1-/-
BALB/c
DO11.10 Rag1-/-
7.13
86.2
1.86
77.7
18
8.15
14.4
78.7
7.94
85.8
1.62
43.2
41
CD4
CD8
B
DP gated
CD5
11.2
0.87
86.8
73.5
17.3
7.41
57.2
32.6
3.17
52
0.06
45.8
13.9
1.03
83
89.7
0.94
7.33
C
CD4SP gated
CD8SP gated
TCR

Figure 3.6 Analysis of the thymic phenotype of *b2m*$^{-/-}$ and MHC-II Δ/Δ mice

B6 control, *b2m*$^{-/-}$ and B6.129-*H2*$^{dlab1\text{-}Ea}$ (MHC-II Δ/Δ) mice were analysed by flow cytometry, for the expression of CD4, CD8, TCR and CD5. (**A**) Density plots show CD4 against CD8 expression for live thymocytes from indicated strains of mice. CD4 SP, CD8 SP and DP gates in *b2m*$^{-/-}$ and MHC-II Δ/Δ mice are based on a WT control. (**B**) Density plots show CD5 against TCR expression on DP thymocytes. (**C**) Density plots show CD5 against TCR expression for CD4 SP and/or CD8 SP populations. Density plots additionally show DP2 and DP3 gates from (**B**). Data in (**A-C**) is representative of ≥3 replicate mice.

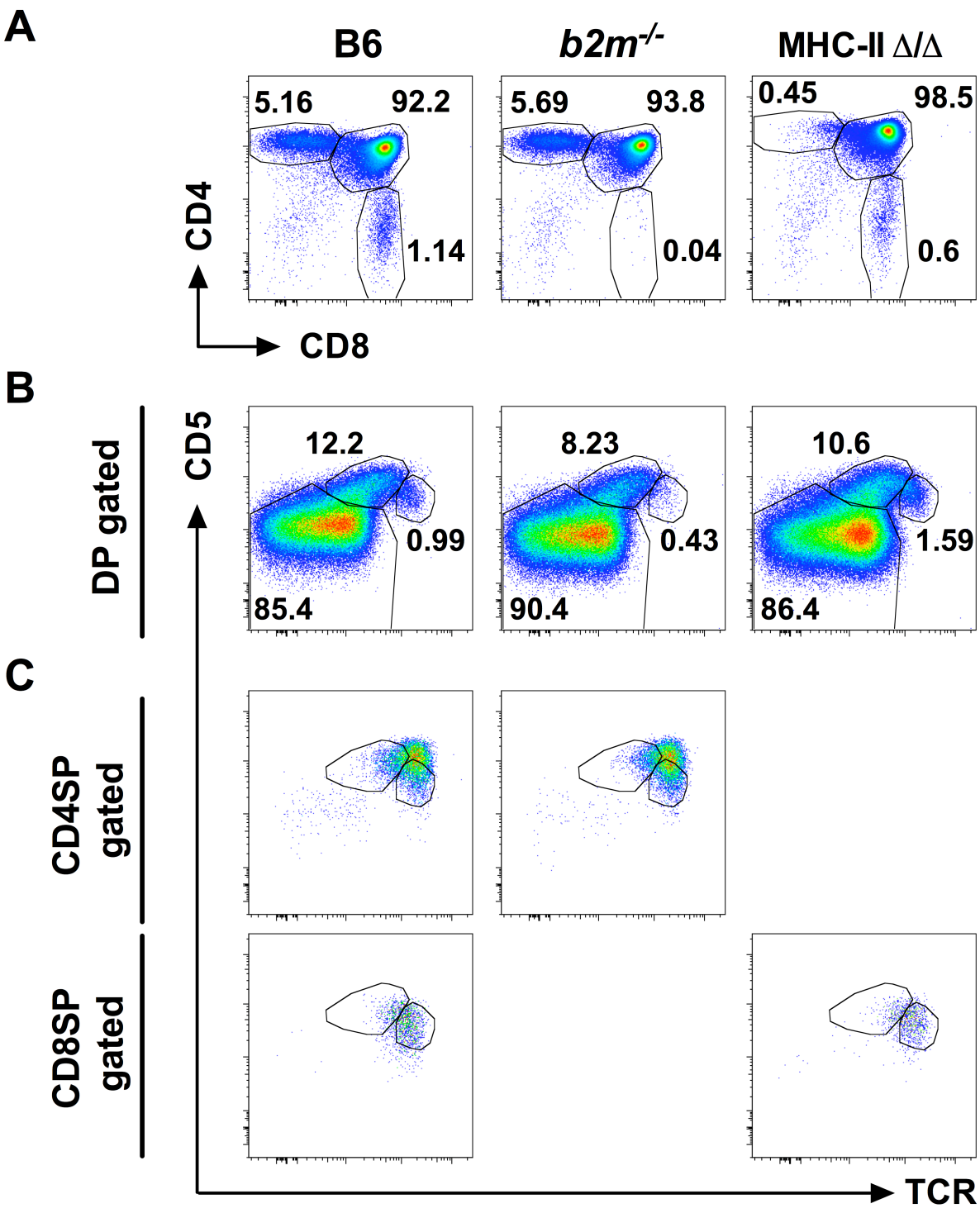

A
B6
b2m-/-
MHC-II Δ/Δ
5.16
92.2
1.14
5.69
93.8
0.04
0.45
98.5
0.6
CD4
CD8
B
DP gated
CD5
12.2
0.99
85.4
8.23
0.43
90.4
10.6
1.59
86.4
C
CD4SP gated
CD8SP gated
TCR

Figure 3.7 DP1-3 populations display a distinct mRNA expression profile

DP1, DP2, DP3, CD4 SP and CD8 SP thymocytes were sorted to high purity (≥95%) from B6 mice. Cellular mRNA was purified and analysed by RT-qPCR. Bar charts show the mean expression of (**A**) Gata3, (**B**) Thpok, (**C**) Runx3 and (**D**) Ctsw relative to Hypoxanthine-guanine phosphoribosyltransferase-1 (Hprt1). Analysis was performed in triplicate wells and error bars represent the sd. Data is representative of ≥3 independent experiments.

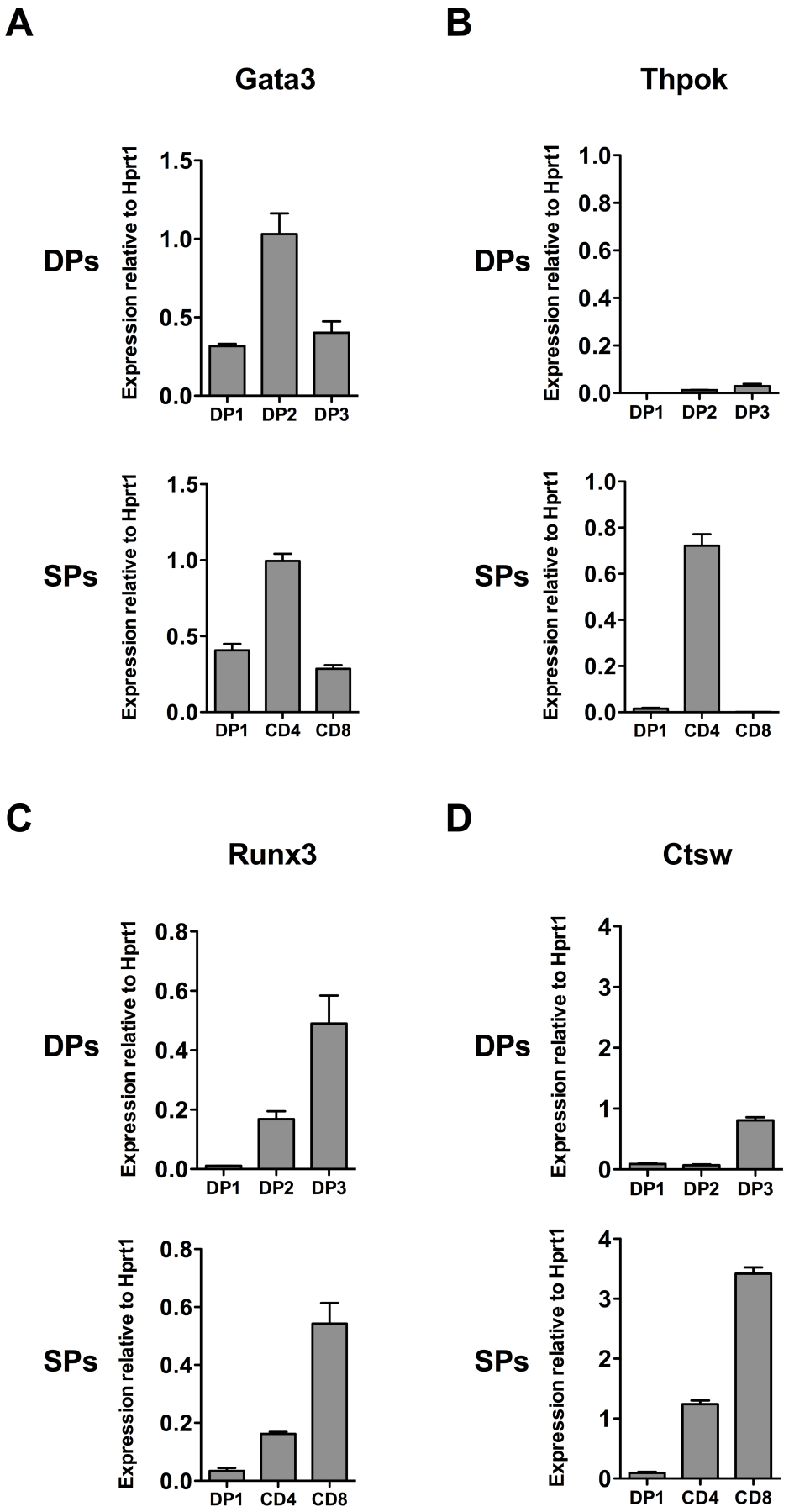

A
Gata3
DPs
Expression relative to Hprt1
1.5
1.0
0.5
0.0
DP1
DP2
DP3
SPs
Expression relative to Hprt1
1.5
1.0
0.5
0.0
DP1
CD4
CD8
B
Thpok
DPs
Expression relative to Hprt1
1.0
0.8
0.6
0.4
0.2
0.0
DP1
DP2
DP3
SPs
Expression relative to Hprt1
1.0
0.8
0.6
0.4
0.2
0.0
DP1
CD4
CD8
C
Runx3
DPs
Expression relative to Hprt1
0.8
0.6
0.4
0.2
0.0
DP1
DP2
DP3
SPs
Expression relative to Hprt1
0.8
0.6
0.4
0.2
0.0
DP1
CD4
CD8
D
Ctsw
DPs
Expression relative to Hprt1
4
3
2
1
0
DP1
DP2
DP3
SPs
Expression relative to Hprt1
4
3
2
1
0
DP1
CD4
CD8

Figure 3.8 Lineage relationships between DP and SP populations

(**A-C**) DP1, DP2 and DP3 thymocytes from B6 CD45.2$^+$ mice were isolated by cell sorting to ≥90% purity, and 1-2x10^6 cells were intrathymically transferred to B6 CD45.1$^+$ recipients. At different days following transfer, CD45.1$^+$ host cells and CD45.2$^+$ donor cells were analysed by flow cytometry for the expression of CD4, CD8, CD5 and TCR. Density plots show CD4 against CD8 and CD5 against TCR on (**A**) DP1, (**B**) DP2 and (**C**) DP3 thymocytes recovered after indicated numbers of days. CD4 against CD8 is shown for total live cells and CD5 against TCR is shown for populations gated as indicated. (**D**) Total thymocytes were isolated from F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2$^+$ mice and 1-2x10^6 cells were transferred intrathymically into B6 CD45.1$^+$ recipients. At different days following transfer, CD45.1$^+$ host cells and CD45.2$^+$ donor cells were analysed by flow cytometry for the expression of CD4, CD8, CD5 and TCR. Density plots of CD5 against TCR (upper row) and CD4 against CD8 (lower row) on transferred F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2$^+$ F5 cells recovered after indicated numbers of days. F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ and F5 *Rag1*$^{-/-}$ mice are shown as controls. Gates shown are based on host populations. (**E**) DP2 and DP3 thymocytes from B6 mice were isolated by cell sorting to ≥90% purity, and cultured for one day at a concentration of 1-10x10^6 cells/mL in complete Dulbecco's modified Eagle's medium (cDMEM) at 37°C. Live cells were analysed by flow cytometry for the expression of CD4, CD8, TCR and CD5. Density plots show CD4 against CD8 on live cells and CD5 against TCR on DP gated cells as indicated. Gates shown are based on uncultured B6 populations. Data is representative of ≥3 replicates per timepoint.

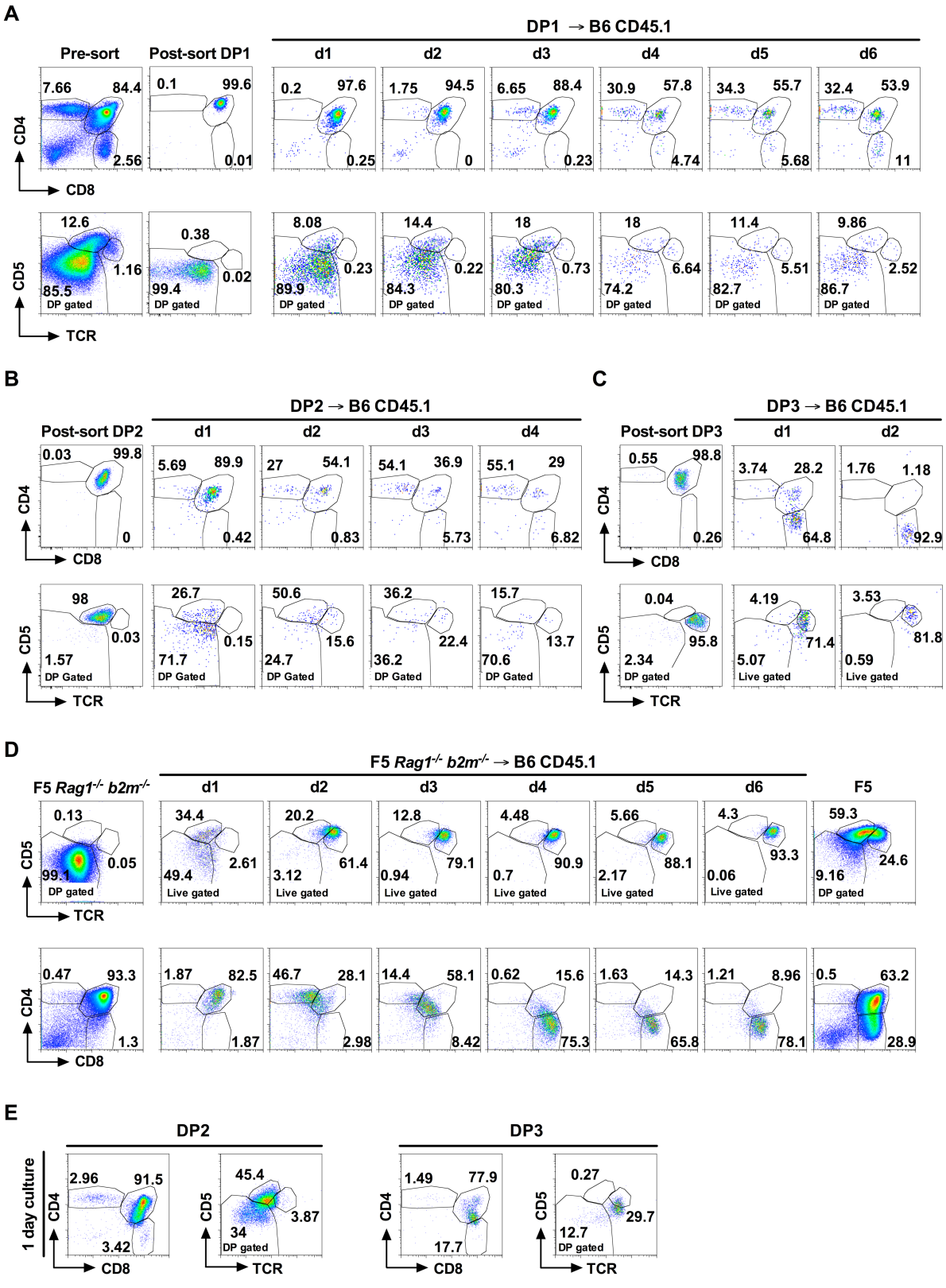

A
DP1 → B6 CD45.1
Pre-sort
Post-sort DP1
d1
d2
d3
d4
d5
d6
CD4
CD8
CD5
TCR
DP gated
B
DP2 → B6 CD45.1
Post-sort DP2
C
DP3 → B6 CD45.1
Post-sort DP3
Live gated
D
F5 Rag1-/- b2m-/- → B6 CD45.1
F5 Rag1-/- b2m-/-
F5
E
DP2
DP3
1 day culture

Figure 3.9 Thpok mRNA expression in thymic subpopulations

TetZap70 mice were fed dox for two or three days. CD4 SP populations were obtained from induced TetZap70 mice, $CD4^{hi}CD8^{lo}$ populations were obtained from B6.129-$H2^{dlab1\text{-}Ea}$ (MHC-II Δ/Δ) mice, and DP1, CD4 SP and CD8 SP populations were obtained from B6 mice, all by cell sorting to ≥90% purity. Total *Rag1*$^{-/-}$ thymocytes were also isolated. Cellular mRNA was purified and analysed by RT-qPCR. (**A**) Density plots show CD4 against CD8 expression on pre- and post-sorted thymocytes for indicated populations. (**B**) Bar chart shows the mean expression of Thpok relative to Hprt1. Analysis was performed in triplicate wells and error bars represent the sd. Data is representative of ≥3 independent experiments. Data for DP1, CD4 SP and CD8 SP B6 control populations are duplicated from **Figure 3.7 B** to enable comparison.

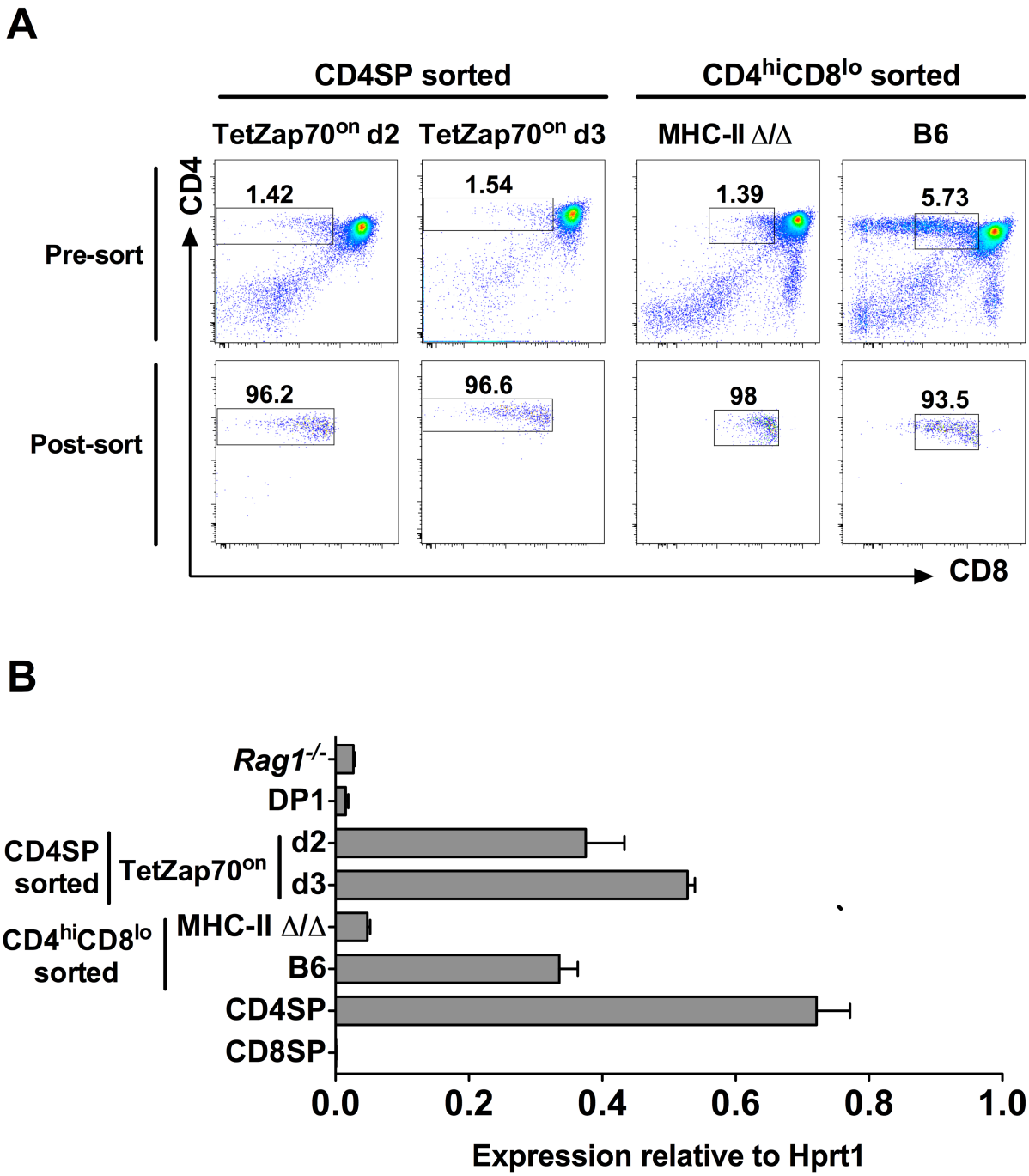

A
CD4SP sorted
CD4hiCD8lo sorted
TetZap70on d2
TetZap70on d3
MHC-II Δ/Δ
B6
Pre-sort
Post-sort
CD4
CD8
1.42
1.54
1.39
5.73
96.2
96.6
98
93.5
B
Rag1-/-
DP1
CD4SP sorted
TetZap70on
d2
d3
CD4hiCD8lo sorted
MHC-II Δ/Δ
B6
CD4SP
CD8SP
0.0
0.2
0.4
0.6
0.8
1.0
Expression relative to Hprt1

Figure 3.10 CD4 SP cells rapidly increase their sensitivity to medullary chemokines following positive selection

B6 mice and TetZap70 mice fed dox for two or three days were analysed for the expression of CCR7 by flow cytometry. (**A**) Histogram shows CCR7 expression on B6 DP1 (grey fill), DP2 (black line), $TCR^{+}CD5^{+}CD4^{+}HSA^{hi}$ (CD4 SP HSA^{hi}, red line) and $TCR^{+}CD5^{+}CD4^{+}HSA^{lo}$ (CD4 SP HSA^{lo}, blue line). (**B**) Histograms show CCR7 expression on B6 DP1 cells (grey fill) and DP2 or CD4 SP HSA^{hi} populations from B6 mice (black line), or TetZap70 mice induced as indicated (red line). Data in (**A-B**) is representative of ≥3 independent experiments. (**C**) DP2 (light grey) and CD4 HSA^{hi} (dark grey) thymocytes from TetZap70 mice fed dox for two days (n=1) or three days (n=3) and B6 controls (n=3) were analysed for their migration towards CCL21 in a chemotaxis assay. The bar chart shows the frequency of migrant thymocytes as a proportion of input. Bars indicate the mean frequency of migration (incorporating two wells per mouse), with error bars corresponding to the sd. The data in (**C**) were obtained by Jara Joedicke.

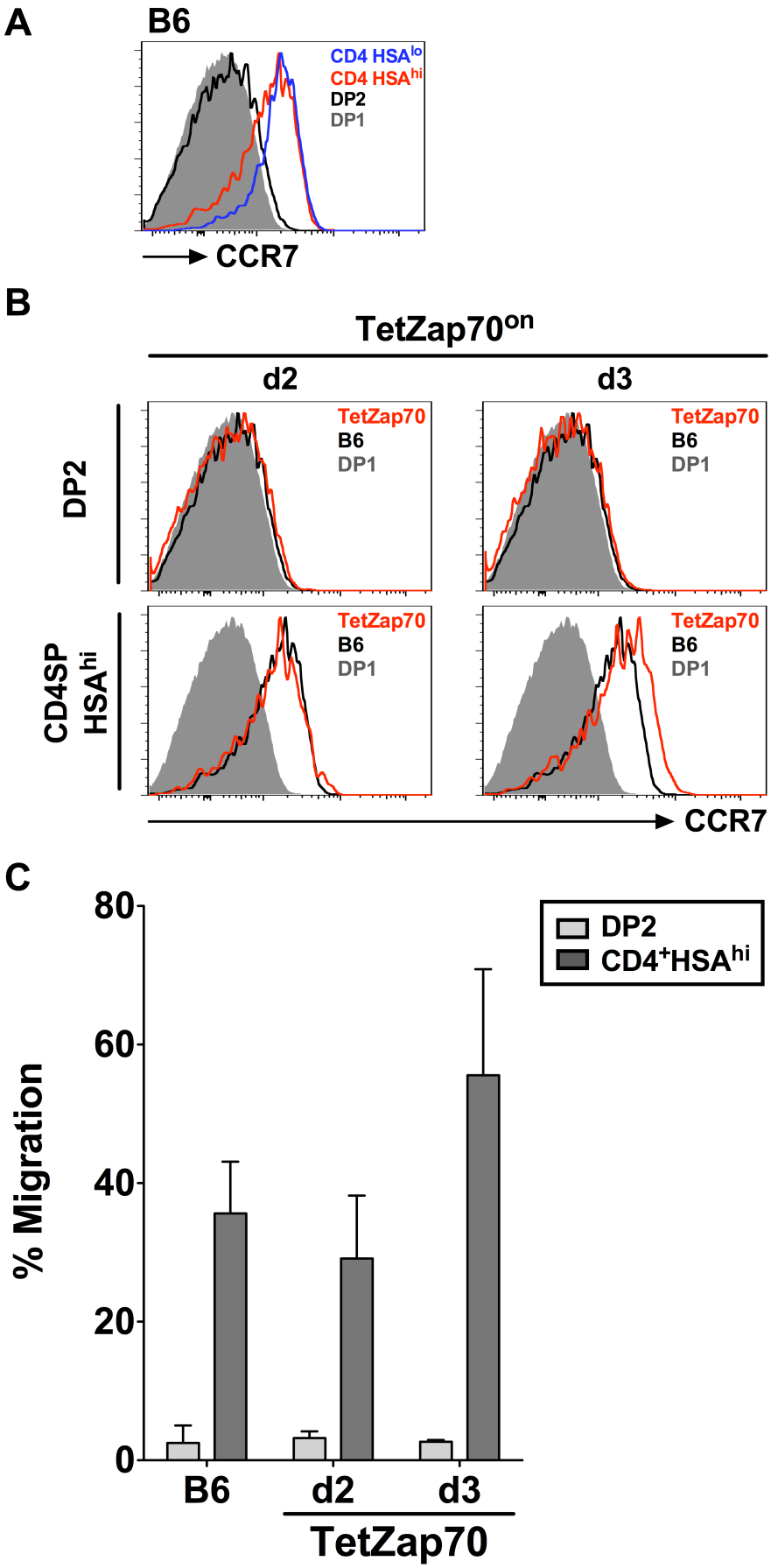

A
B6
CD4 HSA^lo
CD4 HSA^hi
DP2
DP1
CCR7
B
TetZap70^on
d2
d3
DP2
CD4SP HSA^hi
TetZap70
B6
DP1
CCR7
C
% Migration
80
60
40
20
0
DP2
CD4+HSA^hi
B6
d2
d3
TetZap70

Figure 3.11 Asymmetric migration of CD4 and CD8 SP cells to the thymic medulla following positive selection

TetZap70 mice were fed dox for ≤8 days. Thymic sections were prepared from induced TetZap70 mice or B6 controls. Sections were analysed for the expression of CD4 and CD8 by confocal microscopy at 10X and 40X magnification, as indicated. Resultant images show CD4 SP cells (red), CD8 SP cells (green) and DP cells (yellow). On 40X images the thymic cortex (c) and medulla (m) are indicated based on the presence or absence of DP cells respectively. Data is representative of ≥2 replicate mice per timepoint. Preparation of thymic sections and confocal microscopy was performed by Andrea White (University of Birmingham, UK).

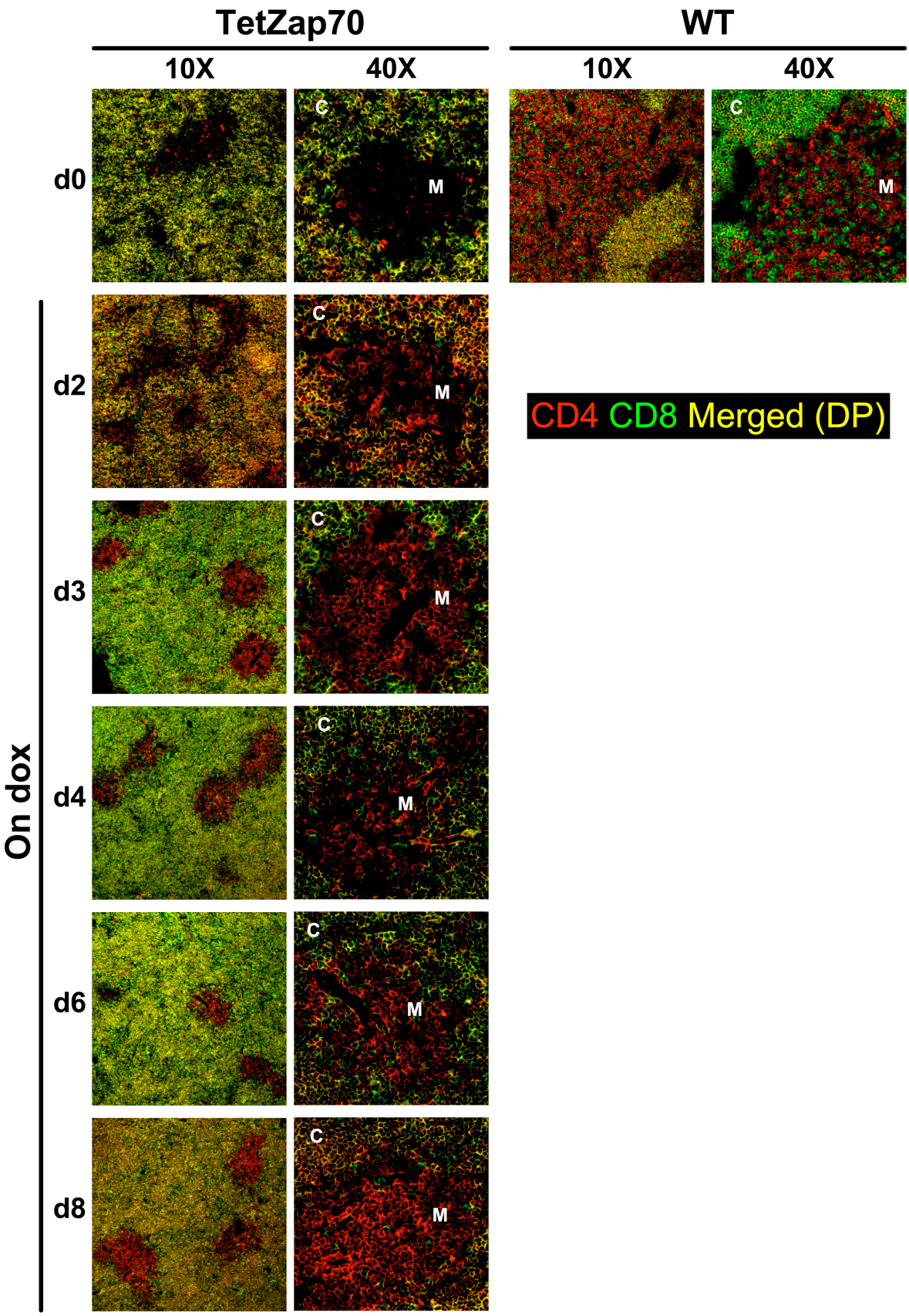
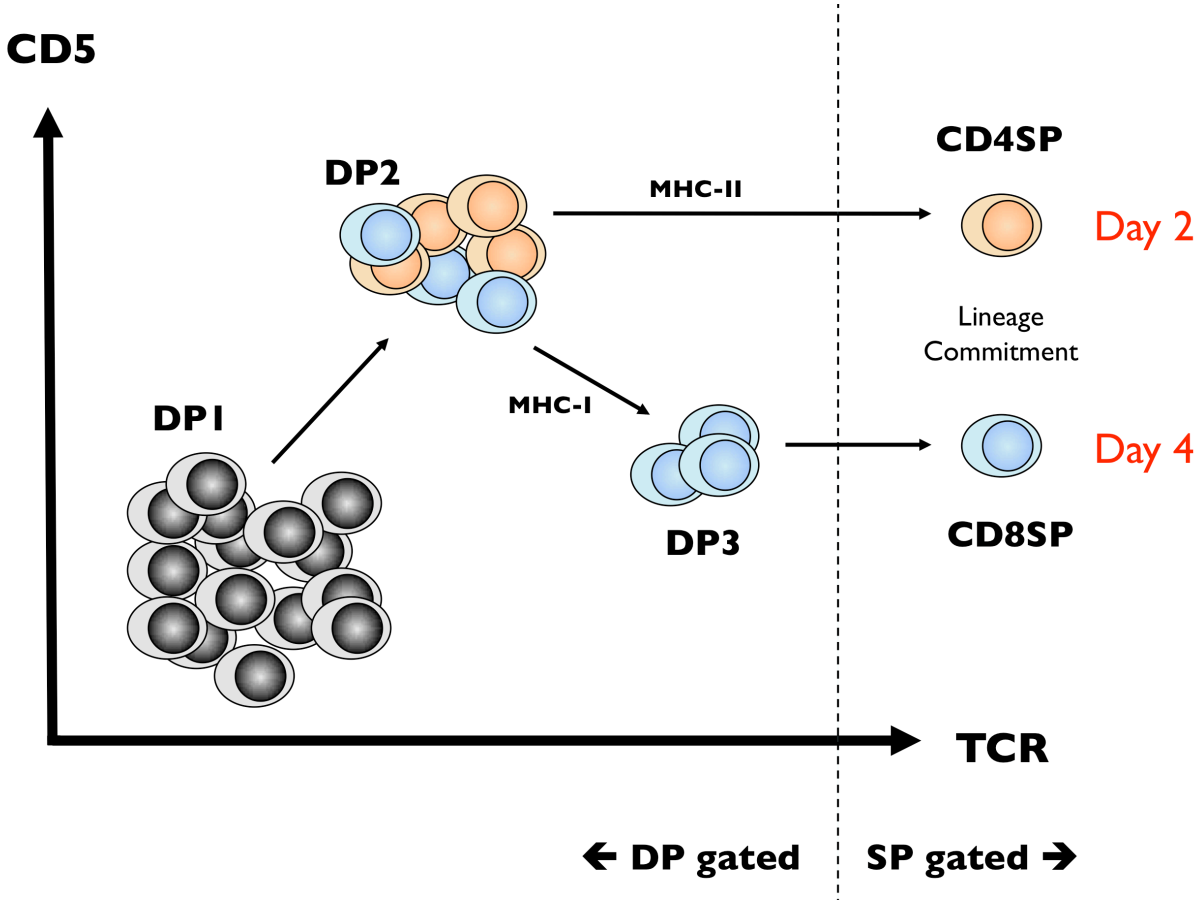

TetZap70
WT
10X
40X
10X
40X
d0
d2
d3
d4
d6
d8
On dox
C
M
CD4 CD8 Merged (DP)

Figure 3.12 A model for the phenotypic progression of positively selecting thymocytes

A model for the phenotypic progression of positively selecting thymocytes. Cells initially reside in the $TCR^{lo}CD5^{lo}$ DP1 population (non-selecting, black). Upon initiation of positive selection through MHC recognition, cells phenotypically transit to the $TCR^{int}CD5^{hi}$ DP2 population. This population contains MHC-I (blue) and MHC-II (orange) restricted thymocytes. MHC-II restricted DP2 thymocytes are the direct precursors of the CD4 SP lineage. MHC-I restricted DP2 thymocytes are the direct precursors of the DP3 population. The DP3 population consists only of MHC-I restricted cells that represent the direct precursors of the CD8 SP lineage. However, the efficient phenotypic progression from DP3 to CD8 SP requires additional intrathymic signals.


CD5
DP2
MHC-II
CD4SP
Day 2
Lineage
Commitment
DP1
MHC-I
DP3
Day 4
CD8SP
TCR
← DP gated
SP gated →

Chapter 4

The Interpretation of the Positive Selection Signal

4.1 Introduction

How positive selection instructs MHC-II restricted T-cells to commit to the CD4 lineage, and MHC-I restricted T-cells to commit to the CD8 lineage remains an ongoing question (Alarcon & van Santen, 2010). TCR signal transduction classically leads to mitogen activated protein kinase (MAPK)/Activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) and Calcium influx/Nuclear factor of activated T-Cells (NFAT) activation (reviewed in (Samelson, 2002)). Evidence from knockout and transgenic mice suggests that such signalling pathways are required for positive selection (reviewed in (Cantrell, 2002)). However, how TCR signalling directly promotes the expression of lineage specifying factors remains to be elucidated (reviewed in (He *et al.*, 2010)). Currently, there are two favoured models addressing how signals through the TCR can result in divergent developmental outcomes. These models focus on divergent signalling mechanisms mediated proximally to the TCR by either the CD4 or CD8 coreceptors.

MHC-II-restricted T-cells receive a relatively stronger positive selection signal then do MHC-I restricted T-cells because CD4 has a higher affinity for Lck than does CD8 (Wiest *et al.*, 1993). The quantitative instructive model proposes that stronger signals instruct CD4 lineage commitment, whereas weak signals instruct CD8 lineage commitment (reviewed in (Hogquist, 2001)). It was found that MHC-II restricted transgenic thymocytes that are CD4-deficient, will develop to the CD8 lineage, presumably because they receive a weaker signal (Matechak *et al.*, 1996). Further evidence for a quantitative instructive model comes from studies utilising neonatal thymic organ culture (NTOC). Culturing cells with agonist stimuli led to CD4 SP lineage generation (Bommhardt *et al.*, 1997), whereas culturing thymocytes with weaker antagonistic stimuli generated CD8 SP cells (Basson *et al.*, 1998). Additionally, constitutive TCR signalling mediated by Lck directed MHC-I-restricted thymocytes to the CD4 lineage, whereas a dominant-negative form of Lck directed MHC-II-restricted thymocytes to the CD8 lineage (Hernandez-Hoyos *et al.*, 2000). Overexpression of Lck in the tetracycline-inducible Lck mice was also found to favour CD4 lineage generation (Legname *et al.*, 2000). However, not all experimental data supports a strictly quantitative model of lineage commitment. For example, mutation of immunotyrosine activation motifs (ITAMs) in TCR-associated CD3ζ-chains reduced TCR signalling sensitivity, but did skew the CD4:CD8 lineage ratio (Holst *et al.*, 2008; Love *et al.*, 2000). Furthermore the generation of mice expressing chimeric CD8α molecules with CD4 cytoplasmic tails that recruit higher levels of Lck, revealed no evidence of lineage redirection of MHC-I signalled thymocytes (Bosselut *et al.*, 2001; Erman *et al.*, 2006). Thus,

although quantitative difference in TCR signalling strength may play a role in the CD4/CD8 lineage decision, the quantitative instructive model cannot explain all of the experimental data.

As an alternative model, it has been proposed that signalling duration may instruct the CD4/CD8 lineage decision, with longer signals promoting CD4 development and shorter signals promoting CD8 development (Yasutomo *et al.*, 2000). Singer and colleagues have suggested that all thymocytes enter a $CD4^{hi}CD8^{lo}$ population following an initial positive selection signal. Thus CD4:spMHC-II signals can be maintained, instructing CD4 commitment, whereas CD8:spMHC-I signals are broken off, leading to CD8 development (Brugnera *et al.*, 2000) (reviewed in (Singer *et al.*, 2008)). Further studies concluded that CD8 lineage specification additionally depended on interleukin (IL)7 signals received at the $CD4^{hi}CD8^{lo}$ stage (Park *et al.*, 2010; Yu *et al.*, 2006). *In vivo* evidence for the kinetic signalling model came from the characterisation of the $CD4^{E8III}$ mouse, which expresses the CD4 coreceptor under control of CD8 expression elements. CD4 coreceptor expression was transcriptionally downregulated following positive selection, resulting in redirection of MHC-II restricted cells to the CD8 lineage (Sarafova *et al.*, 2009). However, it could be argued that these mice exhibit a reduction in signal strength, as a function of time. If CD4 was prematurely terminated, the overall signalling strength interpreted by a cell may be reduced. Furthermore, the presence of the $CD4^{hi}CD8^{lo}$ population in MHC-I-restricted transgenic mouse models has been shown to correlate with TCR signalling strength, suggesting

coreceptor downregulation may function as a salvage pathway for high avidity MHC-I restricted cells (Chan *et al.*, 1998; Correia-Neves *et al.*, 2001). A question that arises is whether the kinetic signalling model and quantitative instructive model are mutually exclusive, or whether aspects of both models may be true (reviewed in (Hogquist, 2001).

Changes in TCR and associated signal-transducing molecules occur during positive selection. TCR expression increases (Havran *et al.*, 1987; Penit, 1990; Shortman *et al.*, 1991), and Zap70 expression is higher in SP relative to DP populations (Chan *et al.*, 1994b; Palacios & Weiss, 2007). Furthermore, CD5 is a negative regulator of TCR signalling and is also modulated in developmentally intermediate populations of positively selecting DPs (**Chapter 3**, **Figure 3.4**) (Tarakhovsky *et al.*, 1995). The temporal separation of developmental cues is a common mechanism by which cellular developmental fate decisions are triggered (reviewed in (Freeman & Gurdon, 2002)). In **Chapter 3** we showed evidence that the CD4/CD8 lineage decision in positively selecting thymocytes was temporally separated. Therefore an important question is whether the temporal modulation of signalling proteins will differentially impact on the signalling requirements for CD4 and CD8 lineage specification.

In this chapter, we have investigated how a selecting T-cell temporally interprets the CD4 versus CD8 lineage commitment signals. Much is known about the essential role of Lck for CD4 lineage development, but we wished to address whether the downstream kinase Zap70 affects the lineage commitment decision. There is evidence that Zap70 expression is upregulated in mature

thymocytes relative to the DP population. We have therefore investigated the kinetics and mechanisms behind this increase. In **Chapter 3**, it was established that the DP2 population contained the direct precursors for the CD4 SP lineage, whereas the DP3 population contained the direct precursors for the CD8 SP lineage. We were interested to address how TCR signalling thresholds may be differentially regulated between the DP2 and DP3 populations, and how this may impact on the interpretation of strong/consistent MHC-II-mediated versus the weak/intermittent MHC-I mediated positive selection signals.

4.2 Results

4.2.1 Zap70 is rate limiting for TCR signal transduction in DP thymocytes

We first wanted to investigate whether Zap70 expression impacted on the positive selection signalling sensitivity, by asking whether Zap70 expression could be a rate-limiting factor in TCR signal transduction. Following CD3-crosslinking, intracellular calcium influx occurs in proportion to the TCR signalling sensitivity (June & Rabinovitch, 1990). Therefore we used the TetZap70 system to investigate whether Zap70 abundance contributes to differences in TCR signalling sensitivity. HuCD2 expression in the TetZap70 mice faithfully reports Zap70 expression in live cells (**Chapter 3**, **Figure 3.1 B**). Hence, we tested whether different levels of HuCD2 expression on TetZap70 thymocytes correlated with a differential calcium influx following CD3-crosslinking. TetZap70 mice were induced to express Zap70 for two days and HuCD2 expression was first examined by flow cytometry. TetZap70 thymocytes exhibited a broad range of HuCD2 expression allowing us to gate on populations exhibiting differential expression (**Figure 4.1 A**, left histogram, gates 1-4). We further found that HuCD2 expression positively correlated with the level of calcium influx (**Figure 4.1 A**, right panel). In addition, this calcium influx was dependent on signals transduced through Zap70, as *Zap70*$^{-/-}$ thymocytes were not capable of fluxing calcium following a CD3-crosslinking stimulus (**Figure 4.1 A**, gate 5). These data suggested that Zap70 expression is a rate-limiting factor during TCR signal transduction in DP thymocytes.

4.2.2 Transgenic Zap70 expression influences the developmental fate of TetZap70 thymocytes

Differences in the positive selection signal strength may instruct the CD4 or CD8 lineage commitment decision (Hernandez-Hoyos *et al.*, 2000; Matechak *et al.*, 1996) (reviewed in (Hogquist, 2001). Zap70 expression was found to be rate-limiting in TCR signal transduction (**Figure 4.1 A**). We hypothesised that Zap70 may play a role in tuning TCR signalling sensitivity in positively selecting cells and thus may influence how TCR signal strength is interpreted. To test this, we first asked whether transgenic Zap70 expression level influenced the developmental fate of TetZap70 thymocytes. DP and SP thymic populations from TetZap70^{on} mice were examined by flow cytometry for the expression of Zap70. We found that relative to non-selecting DP1 cells, the DP2 population expressed a high level of Zap70. In contrast the DP3 population expressed relatively low levels of Zap70, compared to both DP1 and DP2 populations (**Figure 4.1 B**, top panel). Furthermore, Zap70 expression was higher in the mature CD4 SP population relative to the mature CD8 SPs (**Figure 4.1 B**, bottom panel). This was fitting with quantitative instructive model of T-cell development, which suggests that stronger TCR signals promote CD4 SP lineage fate, whereas weaker TCR signals promote CD8 SP lineage fate (reviewed in (Hogquist, 2001)). In B6 thymocytes Zap70 is under control of endogenous regulatory elements as opposed to the TetZap70 transgenic system. We therefore investigated whether the distribution of Zap70 abundance in TetZap70 DP and SP populations was also reflected in B6 thymocytes. Zap70 expression was analysed in different DP and SP populations in B6

thymocytes by flow cytometry. Relative to the DP1 population, we saw an increase in Zap70 expression in DP2 cells. Surprisingly, we saw further increased expression of Zap70 in the DP3 population (**Figure 4.1 C**, top panel). In addition, the mature SP populations had the highest levels of Zap70 expression, which were slightly but consistently higher in the CD8 SP lineage relative to the CD4 lineage (**Figure 4.1 C**, bottom panel). We next confirmed that the increase in Zap70 expression measured by the 1E7.2 anti-Zap70 monoclonal antibody was indeed due to specific differences in Zap70, as opposed to changes in non-specific binding of the antibody in different developmental subsets. Zap70 expression in DP1-3, CD4 SP and CD8 SP subsets from B6 mice was measured by western blotting, using a different monoclonal antibody (99F2) (see **Materials and Methods**, sections **2.3.1** and **2.13.3**). Mirroring the results we see in B6 thymocytes with the 1E7.2 antibody by flow cytometry, we found Zap70 expression was low in the DP1 population of B6 mice (expression of 0.36 relative to GAPDH) (**Figure 4.1 D**, DP1). In contrast we saw a progressive upregulation of Zap70 in the DP2 and DP3 populations (expression of 0.68 and 0.74 respectively, relative to GAPDH) (**Figure 4.1 D**, DP2 and DP3). The CD4 and CD8 SP populations were again found to have the highest levels of Zap70 expression relative to GAPDH (0.87 and 1.11 respectively, relative to GAPDH) (**Figure 4.1 D**, CD4SP and CD8SP). Taken together these data suggest that Zap70 expression in transgenic thymocytes could influence their developmental progression. In contrast, Zap70 expression was progressively upregulated during the development of B6 thymocytes.

4.2.3. Progressive upregulation in TCR signalling sensitivity in B6 thymocytes

We showed in **Figure 4.1 C-D** that Zap70 is upregulated during B6 thymocyte positive selection. In addition, TCR and CD5 expression necessarily differ in these populations (see **Chapter 3**). It was hypothesised that these proteins would influence the intrinsic TCR signalling sensitivity of a positively selecting thymocyte. We next wanted to directly assess whether there were changes in TCR signalling sensitivity in positively selecting DP thymocytes. To do this, we examined calcium influx following CD3-crosslinking, first in B6 DP and CD8 SP populations. We saw relatively low levels of calcium flux in DP1 and DP2 populations, suggesting they are relatively insensitive to TCR signalling (**Figure 4.2 A**). In contrast there was a great increase in TCR signalling sensitivity in the DP3 population and these cells were almost as sensitive to TCR stimulation as B6 CD8 SPs (**Figure 4.2 A**). This suggested there was a upregulation of TCR signalling sensitivity as B6 thymocytes progressively transit through the DP1-3 populations. TetZap70 thymocytes do not upregulate Zap70 expression in DP3 cells (**Figure 4.1 B**), however they express levels of TCR and CD5 identical to B6 mice (see **Chapter 3**). This led us to ask whether differences in the Zap70 expression affected TCR signalling sensitivity in TetZap70 DP3 thymocytes. To investigate this, TetZap70 mice were induced to express Zap70 for four or more days to allow for reconstitution of DP1-DP3 populations. Calcium flux was then induced in thymocytes by CD3-crosslinking and examined by flow cytometry. Similar to B6 thymocytes, TetZap70 thymocytes showed relatively low levels of calcium flux in the DP1 and DP2

populations, suggesting the were relatively insensitive to TCR signalling (**Figure 4.2 B**). In contrast to B6 DP3 thymocytes, the TetZap70 DP3 thymocytes did not show a great increase in TCR signalling sensitivity, as they showed greatly reduced calcium influx compared to B6 CD8 SP controls. Nevertheless the level of calcium influx exhibited by the TetZap70 DP3 population was slightly increased compared with the TetZap70 DP2 population (**Figure 4.2 B**).

We next asked whether TetZap70 and B6 thymocytes had the same potential to influx calcium, independently of TCR signalling. We stimulated cells with ionomycin, a calcium ionophore that induces calcium influx independently of TCR signalling (Liu & Hermann, 1978). Upon treatment with ionomycin, we found no differences in the ability of thymocytes from TetZap70 and B6 mice to flux calcium. This supports the conclusion that differences in calcium influx following CD3-crosslinking between B6 and TetZap70 DP3 populations are due to differences in TCR signalling sensitivity. We additionally noted an increase in the ionomycin induced calcium influx as cells mature through the DP populations, which was identical for both TetZap70 and B6 thymocytes. After approximately 250 seconds, 50% of DP1 cells to show calcium influx following ionomycin treatment, whereas it only took approximately 100 seconds for calcium influx in 50% of DP3 cells (**Figure 4.2 C**). Finally, to test whether differences in the capacity of TetZap70 and B6 DP thymocytes to influx calcium could be due to differential loading of the Indo1 calcium indicator. When we measured Indo1 expression by flow cytometry in unstimulated cells, we found

Indo1 to be identically loaded in DP1-3 populations from both B6 and TetZap70 mice (**Figure 4.2 D**). Taken together this data suggests that a defect in TCR signal transduction underlies the defective ability of TetZap70 DP3 thymocytes to influx calcium following CD3-crosslinking.

4.2.4 No evidence for signalling molecules other than Zap70 contributing to the reduced DP3 signalling sensitivity in TetZap70 thymocytes

Our data suggested that TetZap70 DP3 thymocytes exhibited defective TCR signalling relative to B6 controls. Furthermore, we hypothesised that this reduction in TCR signalling was attributable to differences in the abundance of Zap70. Conceivably other signaling molecules involved in TCR signal transduction may be differently regulated in TetZap70 compared to B6 thymocytes and thus impact on differences in TCR signalling between these mice. To investigate this we compared the expression levels of molecules involved in TCR signal transduction between DP1-3 populations, in TetZap70 mice that had been induced to express Zap70 for four or more days and in B6 control mice. We first looked at the expression of the TCR proximal kinases Lck that is upstream of Zap70 (Iwashima *et al.*, 1994) and the related and partially redundant Fyn proto-oncogene (Fyn) (Appleby *et al.*, 1992). Both Lck and Fyn were identically expressed in DP1-3 thymocyte populations of TetZap70 mice compared to B6 controls (**Figure 4.3**). We also analysed the expression of linker of activated T-cells (LAT) and Src homology 2 (SH2) domain containing leukocyte protein of 76kDa (Slp76), adaptor proteins that are direct downstream targets of Zap70 (Wardenburg *et al.*, 1996; Zhang *et al.*, 1998). Both LAT and

Slp76 were also expressed identically in DP1-3 populations of TetZap70 mice compared to B6 controls (**Figure 4.3**). Finally we looked at a distal MAPK family member extracellular regulated kinase (Erk)2, which is activated by TCR signalling and is downstream of LAT and Slp76 (reviewed in (Samelson, 2002)). We found no differences in Erk2 expression in the DP populations of TetZap70 mice compared to B6 controls (**Figure 4.3**). In summary, we found no evidence that signalling proteins other than Zap70 contributed to a TCR signalling defect in TetZap70 thymocytes. However we cannot rule out that other signalling proteins, whose expression we have not examined, contribute to this difference.

4.2.5 Increased TCR signalling sensitivity is essential for CD8 SP development

TetZap70 DP3 cells have a lower level of Zap70 expression relative to B6 DP3s (**Figure 4.1**), and this correlates with a defect in TCR signalling in the TetZap70 DP3 population (**Figure 4.2**). The DP3 population contained the direct precursors for the CD8 SP lineage, which additionally depended on intrathymic signalling (**Chapter 3**, **Figure 3.8 C, E**). Therefore, we investigated whether the defect in TCR signalling sensitivity in TetZap70 DP3 thymocytes had an impact on the continuing maturation of these cells to the CD8 SP lineage. The TetZap70 mice were crossed with F5 *Rag1*$^{-/-}$ mice that express an MHC-I restricted TCR transgene on a *Rag1*$^{-/-}$ background, giving F5$^{+/-}$ *Rag1*$^{-/-}$ *Zap70*$^{-/-}$ *Zap70*^{Tre} *rtTA*^{HuCD2} (refer to **Materials and Methods**, section **2.1**, F5 TetZap70 hereon). This allowed us to assess the affect of transgenic Zap70 on a monoclonal population of positively selecting MHC-I restricted thymocytes. To

first address whether transgenic Zap70 expression permitted thymocyte maturation beyond positive selection in the F5 system, we analysed the thymic phenotype of F5$^{+/-}$ *Rag1*$^{-/-}$, F5$^{+/-}$ *Rag1*$^{-/-}$ *Zap70*$^{-/-}$ and F5 TetZap70 mice that were fed dox for ≥7 days (F5 TetZap70^{on} hereon), by flow cytometry. The post-selection DP2 and DP3 populations in addition to CD8 SPs were found in F5$^{+/-}$ *Rag1*$^{-/-}$ mice (**Figure 4.4 A**, left column). In contrast, the F5 *Rag1*$^{-/-}$ *Zap70*$^{-/-}$ thymocytes were arrested at the DP1 stage; showing much reduced frequencies of DP2, DP3 and CD8 SP thymocytes (**Figure 4.4 A**, middle column). When we examined the HuCD2^{+} thymocytes from F5 TetZap70^{on} thymi, we found that transgenic Zap70 expression facilitated continued thymic maturation, as DP2, DP3 and CD8 SP thymic populations were present. However, transgenic Zap70 expression abated CD8 SP development, as this population was reduced in frequency relative to intact F5$^{+/-}$ *Rag1*$^{-/-}$ mice (**Figure 4.4 A**, right column). To pinpoint the developmental block in CD8 SP development, we compared the ratio between precursor and progeny thymic populations in a cohort of F5$^{+/-}$ *Rag1*$^{-/-}$ and F5 TetZap70^{on} mice. There was no significant difference in the DP3:DP2 ratio between F5$^{+/-}$ *Rag1*$^{-/-}$ and F5 TetZap70^{on} mice (**Figure 4.4 B**, left). In contrast, we saw a significant reduction in the ratio of CD8 SP:DP3 cells in the F5 TetZap70^{on} mice compared to F5$^{+/-}$ *Rag1*$^{-/-}$ controls ($p \leq 0.01$, Student's t-test: F5$^{+/-}$ *Rag1*$^{-/-}$ n=2, F5 TetZap70 n=3) (**Figure 4.4 B**, right). This data suggested that the failure to upregulate Zap70 in the DP3 population results in a significant block in positive selection between the DP3 and CD8 SP populations.

4.2.6 Reduced function but normal regulation of Zap70 affects the CD4 and CD8 SP lineages equally

TetZap70 thymocytes fail to upregulate Zap70 in the DP3 and SP populations. This affected the CD8 lineage development but had a much lesser effect on CD4 SP generation (**Figure 4.4 B**) (Saini *et al.*, 2010). We therefore hypothesised that an overall reduction in Zap70-dependent signalling may specifically affect the development of the CD8 SP population. To investigate this, we examined the thymic phenotype of SKG mice, which have a W163C mutation in the carboxy-terminal SH2 domain of Zap70, resulting in a hypomorphic allele (Sakaguchi *et al.*, 2003). We first investigated whether positive selection was affected in a cohort of mice. There was no difference between BALB/c and SKG mice in terms of thymic cellularity (**Figure 4.5 A**), however there was a significant reduction in the $TCR^{hi}CD5^{hi}$ population (**Figure 4.5 B**) ($p \leq 0.001$, Student's t-test BALB/c n=10, SKG n=10). This suggested that there was a defect in positive selection of SKG thymocytes, fitting with previous reports (Sakaguchi *et al.*, 2003). Examination of the thymic phenotype of SKG mice showed reduced frequencies of both CD4 and CD8 SP populations relative to BALB/c controls (**Figure 4.5 C**, top row). In addition, there was a relative reduction in the frequency of DP2 and DP3 populations, with an overrepresentation of DP1 cells in the SKG mice (**Figure 4.5 C**, bottom row). We next asked whether the SKG mutation affected peripheral T-cell homeostasis. There was a significant reduction in T-cell numbers in the LN of SKG mice relative to BALB/c controls ($p \leq 0.001$, Student's t-test BALB/c n=10, SKG n=10) (**Figure 4.5 D**). In addition, both CD4 and CD8 lineages were

affected, as both lineages showed reduced percentages as a frequency of lymphocytes in SKG mice, relative to BALB/c controls (**Figure 4.5 E**). Our data suggested that the SKG mutation had an effect on both CD4 and CD8 lineage development and homeostasis. This led us to ask whether either the CD4 or CD8 lineages were more severely affected by the reduction in Zap70 expression. We compared the CD4:CD8 ratio in thymi and LNs of a cohort of BALB/c control and SKG mice. There was found to be no significant difference in the CD4:CD8 ratio in either the thymi or LNs of SKG mice (**Figure 4.5 F**) (CD4/CD8: Thymus: BALB/c=5.99±0.93 n=10, SKG=6.40±0.64 n=10, LN: - BALB/c=2.51±3.00 n=5, SKG=2.17±2.71 n=5). This implied that a simple reduction in Zap70 function could not account for a specific defect in CD8 lineage development in TetZap70 mice.

In TetZap70 mice, the transgenic expression of Zap70 resulted not just in a reduction of Zap70 in more mature thymic populations, but also in dysregulated Zap70 expression during T-cell ontogeny (**Figure 4.1 B**). We therefore asked whether the SKG mice also showed differences in the pattern of Zap70 expression. Zap70 expression was examined in the DP1-3, CD4 and CD8 SP populations of SKG mice and BALB/c controls. Relative to the DP1 population, we saw an increase in Zap70 expression in DP2 cells from both BALB/c controls and SKG mice. Also there was a further increase in the expression of Zap70 in the DP3 population (**Figure 4.5 G**, top panel). The mature SP populations of BALB/c and SKG mice had the highest levels of Zap70 expression (**Figure 4.1 G**, bottom panel). This was despite the consistently

lower expression of Zap70 in SKG mice compared to equivalent populations in BALB/c mice (**Figure 4.1 G**). Taken together, this data suggests that SKG mice have an overall defect in positive selection, which affects both the CD4 and CD8 lineages equally. In addition, SKG mice show normal developmental patterns of Zap70 regulation, although overall abundance and function is reduced.

4.2.7 Zap70 and CD5 are transcriptionally regulated during positive selection

TetZap70 mice exhibit a specific defect in CD8 lineage development relative to the CD4 lineage (**Figure 4.2 B-C, Figure 4.4**) (Saini *et al.*, 2010), which correlated with lower Zap70 expression in DP2, DP3, CD4 SP and CD8 SP populations relative to a B6 control. However, SKG thymocytes also have defects in TCR signalling, which affects both CD4 and CD8 lineage development equally (**Figure 4.5**) (Sakaguchi *et al.*, 2003). We hypothesised that the CD4/CD8 lineage decision may be more dependent on the regulation of Zap70, rather than the overall abundance. We therefore investigated the mechanism controlling Zap70 regulation in WT cells during positive selection. To do this, we sorted DP1-3, CD4 SP and CD8 SP thymocytes from thymi of B6 mice and examined the Zap70 gene expression in these populations by RT-qPCR. We additionally examined expression of Zap70 in total *Rag1*$^{-/-}$ thymocytes that are blocked at the DN3 stage of development as a control (Godfrey *et al.*, 1994; Mombaerts *et al.*, 1992b). DP1 cells expressed more Zap70 mRNA than total *Rag1*$^{-/-}$ thymocytes, consistent with previous studies (Chan *et al.*, 1994b; Palacios & Weiss, 2007). However, when we looked in the

DP2 and DP3 populations, we found there to be an approximately 2-fold increase in the amount of Zap70 mRNA. Zap70 mRNA levels in CD4 and CD8 SP populations were found to be similar level to that of DP1 cells, suggesting that Zap70 mRNA is reduced upon completion of positive-selection (**Figure 4.6 A**). This contrasts with Zap70 protein expression, which highest in SP populations of WT mice (**Figure 4.1 C**). Thus our data suggests that post-transcriptional mechanisms may maintain high Zap70 protein expression in SP cells. These mechanisms may include increased translation efficiency, or decreased Zap70 protein turnover.

We also investigated CD5 mRNA expression, as it has been suggested that CD5 is transcriptionally regulated during thymic development (Yang *et al.*, 2004). Similarly to Zap70, CD5 was found to be expressed at background levels in *Rag1*$^{-/-}$ thymocytes and expressed at low levels in TetZap70 DP1 cells. In addition CD5 mRNA showed an approximately 6-7 fold increase in DP2 cells compared to DP1. However, in contrast to Zap70 mRNA expression patterns, DP3 cells exhibited a relatively low level of CD5 mRNA expression, similar to that of the DP1 population. CD5 mRNA expression was approximately 2-3 fold increased over DP1 in the CD4 SP population, whereas it was expressed at a similar level to the DP1 cells in the CD8 SP population (**Figure 4.6 B**). Taken together, these data suggest that both Zap70 and CD5 are under transcriptional regulation during positive selection, although both show slightly different expression patterns, particularly in the DP3 population. CD5 expression decreases in the DP3 population despite the finding that DP3 cells are more

sensitive to TCR signals than DP2 cells (**Figure 4.6 B**, **Figure 4.2 A**). Thus our data also implies that CD5 is regulated independently of TCR signalling in DP3 cells.

4.2.8 Conserved transcription factor binding sites exist upstream of the *Zap70* gene

Analysis of Zap70 mRNA levels suggested that *Zap70* was transcriptionally regulated during positive selection. Genetic elements controlling gene expression are often conserved across species (Levy *et al.*, 2001). We therefore asked whether any evolutionary conserved regions (ECRs) were associated with the *Zap70* gene. To do this we compared the *Zap70* sequence and approximately 5-10 kilobases (kb) upstream and downstream of the gene. We did this using a comparative genomics approach, comparing human and mouse *Zap70* sequences using the ECR browser tool (available online at http://ecrbrowser.dcode.org/, accessed September 2010). Such analysis revealed a number of noncoding ECRs associated with *Zap70*. One notable region was approximately 250 base pairs (bp) in length and was located proximally upstream of the transcriptional start site (**Figure 4.7 A**). We hypothesised that this represented conservation within the *Zap70* promotor. We further examined this by asking whether there were any evolutionary conserved transcription factor binding sites in the putative promotor sequence. When we compared the mouse and human sequence to look for evolutionary conserved transcription factor binding sites, we found amongst others an E-twenty six (Ets) site, an E-box, an NFAT binding site and a cAMP response element-binding

(CREB) binding site within the ECR proximal to the *Zap70* gene transcriptional start site (**Figure 4.7 B**, left). Furthermore, direct alignment of mouse and human genomes showed that the conserved transcription factor binding sites within this region were almost entirely conserved, or have incorporated only transition mutations (**Figure 4.7 B**, right). Taken together, this analysis shows that a noncoding ECR containing conserved transcription factor binding sites, exists upstream of the *Zap70* gene.

4.2.9 *in vitro* models of positive selection suggest that TCR signal regulates Zap70 expression

TCR signalling has been directly implicated in the regulation of factors binding to Ets, E-box, NFAT and CREB binding sites (reviewed in (Macian, 2005; Murre, 2005; Shaywitz & Greenberg, 1999)). Conserved binding sites for these factors are present immediately upstream of *Zap70* in a non-coding ECR (**Figure 4.7**). Furthermore, it has been reported that Zap70 expression is upregulated on mature T-cells following antigenic stimulation (Chandok *et al.*, 2007). We therefore wanted to investigate whether TCR signals could directly modulate Zap70 expression in DP thymocytes. To test this, we treated DP1 cells *in vitro* using a combination of phorbol-12-myristate 13-acetate (PMA) and ionomycin, at concentrations known to mimic positive selection signalling (Ohoka *et al.*, 1996). We first purified DP1 cells, by depleting B6 thymocytes of $CD3^+$, $CD69^+$ and $CD25^+$ cells. This enabled us to enrich DP1 cells to approximately 90% purity (**Figure 4.8 A**). We cultured the enriched DP1 cells with PMA and ionomycin for 14 hours, before washing out the reagents and allowing the cells

to rest for the remainder of the culture time. Cells were stimulated with PMA or ionomycin alone, or in the absence of reagents as controls. After 22 hours, the combined PMA and ionomycin stimulation induced downregulation of both CD4 and CD8 coreceptors, which was not seen in the absence of one or both reagents (**Figure 4.8 B**, top row). By 46 hours, the PMA and ionomycin treated DP1 cells were phenotypically CD4 SP. In contrast, control cells stimulated with PMA or ionomycin alone, or with neither reagent, remained phenotypically DP (**Figure 4.8 B**, bottom row). CD69 is upregulated on positively selecting thymocytes, downstream of TCR signalling (Swat *et al.*, 1993). We next asked whether the PMA and ionomycin treatment in our protocol induced CD69 upregulation. CD69 was highly expressed on the PMA and ionomycin treated cells by 22 hours, but not on controls treated with either or none of these reagents. Furthermore CD69 was reduced in PMA and ionomycin treated cells by 46 hours (**Figure 4.8 C**, left column). This data suggested that PMA and ionomycin treatment mimicked the positive selection TCR signal.

We next asked if mimicking the positive-selection TCR signal would also induce Zap70 upregulation. We found a large increase in Zap70 expression in PMA and ionomycin treated DP1 thymocytes by 22 hours, which was maintained after 46 hours. In contrast, untreated cells or treatment with either reagent alone did not upregulate Zap70 (**Figure 4.8 C**, right column). This suggests that the TCR signal may directly regulate Zap70 expression in DP thymocytes. We wanted to determine whether the upregulation of Zap70 induced by PMA and ionomycin treatment depended on protein transcription and/or translation.

To investigate this, actinomycin D (ActD) (an inhibitor of transcription (Reich, 1964)) or cyclohexamide (Chx) (an inhibitor of translation (Obrig *et al.*, 1971)) were added to PMA and ionomycin stimulated DP1 thymocytes. Addition of either ActD or Chx reduced but did not prevent CD4 and CD8 coreceptor downregulation after 22 hours (**Figure 4.8 D**, density plots). We found that treatment with either inhibitor blocked Zap70 upregulation and furthermore that Zap70 abundance was reduced relative to non-treated controls (**Figure 4.8 D**, histogram). These data suggested that TCR signalling controls Zap70 expression, through a mechanism that is dependent on both transcription and translation.

TCR signalling activates divergent downstream pathways including MAPKs (implicated in Ets and E-protein regulation), NFAT and CREB (reviewed in (Murre, 2005; Samelson, 2002; Shaywitz & Greenberg, 1999)). To investigate if these downstream pathways contribute to Zap70 upregulation, we first treated cells with the MAPK inhibitor U0126 (an inhibitor of MAPK/Erk kinase (MEK) (Favata *et al.*, 1998)) and the CREB inhibitor Kn93 (an inhibitor of Calcium/calmodulin-dependent protein kinase (CamK)-II (Sumi *et al.*, 1991), which lies upstream of CREB activation (Sheng *et al.*, 1991)), along with PMA and ionomycin. Addition of either U0126 or Kn93 to the culture medium had no effect on Zap70 upregulation (**Figure 4.8 E**), suggesting that MAPK and CREB signalling pathways may not be important for Zap70 upregulation.

We asked whether NFAT signalling was important by treating cells with varying concentrations of cyclosporin A (CsA) (an inhibitor of calcineurin, which is

essential for NFAT activation (McCaffrey *et al.*, 1993)), along with PMA and ionomycin. Increasing concentrations of CsA correlated with decreased CD4 and CD8 coreceptor downregulation at 22 hours following PMA and ionomycin treatment (**Figure 3.8 F**). In addition, increasing CsA concentrations also reduced Zap70 upregulation in PMA and ionomycin treated cells (**Figure 3.8 G**). To exclude a potential confounding role for off-target effects of CsA, we cultured cells with another NFAT inhibitor FK506 (McCaffrey *et al.*, 1993). In agreement with results following CsA treatment, FK506 treated cells also failed to upregulate Zap70 in PMA and ionomycin cultures (**Figure 3.8 F**). Taken together, these data represent *in vitro* evidence suggesting that TCR signalling regulates *Zap70* gene transcription in a calcineurin/NFAT-dependent, but MEK and CamK-II-independent manner.

4.2.10 The positive selection signal modulates Zap70 expression during positive selection *in vivo*

We next wanted to test whether Zap70 expression could be modulated downstream of TCR signalling *in vivo*. F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ mice show DP1 arrested thymic development, as they lack the β_2m selecting ligand for the MHC-I restricted F5 TCR (**Chapter 1**, section **3.2.4**). We therefore investigated the effects of TCR stimulation on DP1 arrested F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes, following intraperitoneal (I.P.) administration of anti-CD3 antibody, along with a low dose of anti-CD28 on F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes. We saw no changes in expression of the CD4 or CD8 coreceptors on thymocytes 24 hours after injection (**Figure 4.9 A**, top row). However, we did note the development of a

DP2 population in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ mice injected with anti-CD3/CD28, that was not present in controls (**Figure 4.9 A**, bottom row). The DP2 population showed increased abundance of CD69 relative to anti-CD3/CD28 injected DP1 cells, or non-injected controls (**Figure 4.9 B**). This suggested that anti-CD3/CD28 treatment induced TCR signalling in thymocytes. We next asked whether Zap70 expression was increased in the DP2 cells in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ mice, following I.P. injection of anti-CD3/CD28. We observed a consistent increase in Zap70 expression in this DP2 population, as compared with DP1 populations in anti-CD3/CD28 treated mice or non-injected controls (**Figure 4.9 C**). Taken together, these data suggest that TCR signalling *in vivo* upregulates Zap70 abundance in DP thymocytes.

Finally, we wanted to test whether quantitative differences in the positive selection signal affected the regulation of Zap70. To do this F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} thymocytes were injected directly into thymi of F5 *Rag1*$^{-/-}$ CD45.1^{+} or B6 CD45.1^{+} hosts. This alleviated the block in positive selection, as both hosts expressed the MHC-I selecting ligand. F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} cells transferred to F5 *Rag1*$^{-/-}$ donors must compete with a monoclonal population of T-cells for positive selection ligands. In contrast, we hypothesised that transfer to B6 hosts results in a reduction in intraclonal competition, and thus may improve the positive selection signal, as shown by others (Canelles *et al.*, 2003). To investigate the effects of reducing the clonal competition on the positive selection signal, CD69 expression was examined on transferred cells after two days. We found that F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ cells transferred to B6 hosts

displayed higher CD69 abundance relative to F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes transferred to F5 *Rag1*$^{-/-}$ hosts (**Figure 3.9 D**), confirming that reducing clonal competition increases the positive selection signal strength or efficiency. We next investigated whether the kinetics of Zap70 upregulation differed in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes following intrathymic transfer to B6 or F5 *Rag1*$^{-/-}$ hosts. Cells transferred to B6 hosts upregulated Zap70 with more rapid kinetics and to a greater extent than those transferred to F5 *Rag1*$^{-/-}$ hosts (**Figure 3.9 E**). However, despite the increased kinetics and abundance of Zap70 upregulation in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes transferred to B6 hosts, B6 host CD8 SPs still expressed relatively higher levels of Zap70. This is in keeping with the intrinsically low avidity of the F5 TCR for spMHC (**Figure 3.9 E**, host CD8SP) (Ge *et al.*, 2004). When taken together, the evidence presented in this chapter suggests that Zap70 expression is tuned in response to the TCR signalling strength during positive selection.

4.3 Discussion

We have identified a novel developmental role for Zap70 in modulating the TCR signalling threshold during positive selection. Zap70 was found to be a rate-limiting factor for TCR signal transduction in DP thymocytes. In addition, the abundance of Zap70 correlated with the developmental fate decision taken by TetZap70 thymocytes. In contrast, when we examined WT mice, we found that Zap70 was progressively upregulated along with maturity throughout positive selection. TetZap70 thymocytes exhibited a block in CD8 SP development, whereas the CD4 SP lineage was less affected (**Chapter 1**, **Figure 3.1 G**) (Saini *et al.*, 2010). We therefore hypothesised that the CD8 SP lineage developmental defect resulted from either a reduction in function of Zap70 expression, or dysregulated developmental upregulation. We found that a simple reduction in Zap70 function affected both CD4 and CD8 lineages equally, and thus argue that developmental dysregulation of Zap70 underlies the CD8 SP developmental defect. Finally we have provided evidence that the positive selection TCR signal directly influenced Zap70 expression levels. Therefore, we suggest a model whereby the external signal mediated by MHC molecules influences the internal sensitivity of the TCR. CD4 and CD8 dependent signals are likely to mediate strong/consistent versus weak/intermittent signals respectively. In addition we have shown in **Chapter 3** that CD4 and CD8 lineage development occurs with temporal distinction. A feedforward loop mechanism, whereby TCR signals promote increased signalling sensitivity of the TCR, may enable greater resolution of

strong/consistent MHC-II-mediated signals at early times and weak/intermittent MHC-I-mediated signals at later times. Thus by temporally separating the CD4/CD8 lineage commitment decision, signals through the TCR may be conducive to triggering divergent developmental fates.

We first found that Zap70-mediated signal transduction is rate-limiting for TCR signalling during positive selection. When we analysed calcium influx following CD3-crosslinking in DP gated TetZap70 cells, we found that increased calcium influx correlated with increased HuCD2 (and thus Zap70) expression in thymocytes (**Figure 4.1 A**). Furthermore, WT Zap70 was developmentally increased throughout positive selection (**Figure 4.1 C**). In addition, TetZap70 DP3 thymocytes were phenotypically indistinguishable to those of WT in all aspects examined, except with regards to Zap70 abundance (**Chapter 3**, **Figure 3.1 D** and **Figure 4.2 C, Figure 4.3**). This difference in Zap70 in TetZap70 and WT DP3 populations correlated with reduced sensitivity to anti-CD3 crosslinking of TetZap70 thymocytes (**Figure 4.2 A**-**B**). Other researchers have shown that WT SP thymocytes show increased calcium influx following CD3-crosslinking, relative to DPs (Davey *et al.*, 1998; Finkel *et al.*, 1987; Havran *et al.*, 1987; Hedin *et al.*, 1995). Our results suggest that Zap70 expression functionally contributes to this developmental increase in TCR signalling sensitivity.

Zap70 expression levels were found to influence the developmental fate decision of transgenic thymocytes. Whereas high levels of Zap70 expression were found in DP2 and CD4 SP thymic populations in TetZap70^{on} mice, we

found a relatively lower abundance of Zap70 in DP3 and CD8 SP compartments (**Figure 4.1 B**). Such a finding is entirely reconcilable with the quantitative instructive model of T-cell development, which states that strong avidity signals promote CD4 SP development, whereas weak signals promote CD8 SP development (Hernandez-Hoyos *et al.*, 2000; Matechak *et al.*, 1996) (reviewed in (Hogquist, 2001)). In TetZap70 mice, higher levels of Zap70 in DP thymocytes may permit stronger signal transduction leading to CD4 development. The remaining cells with less transgenic Zap70 may be unable to transduce sufficiently strong signals to instruct CD4 lineage development and may be predisposed to CD8 SP lineage development. This is consistent with the low level of Zap70 observed in TetZap70 DP3 cells (**Figure 4.1 B**).

Thymic Zap70 expression in WT mice sharply contrasted with that seen in the TetZap70 mice. We found a progressive upregulation of Zap70 throughout the DP populations in WT mice and showed that Zap70 is expressed at the highest levels in thymic SP populations (**Figure 4.1 B**, **D**). The loss of developmental regulation of Zap70 in TetZap70 mice may therefore reveal underlying signalling requirements, which trigger CD4 SP versus DP3 progression from the DP2 precursor population. Such a conclusion assumes that lower Zap70 expression in TetZap70 DP3 thymocytes reflects a selective event, as opposed to resulting from a downregulation of Zap70. We favour the hypothesis that low Zap70 expression in DP3 cells represents a selective event, as there is no reported evidence for a CD8 lineage-specific downregulation in other tetracycline inducible systems (Labrecque *et al.*, 2001; Legname *et al.*, 2000). However,

this could be more conclusively determined by following cell fate of $Zap70^{hi}$ and $Zap70^{lo}$ cells, following intrathymic transfer to dox fed hosts.

We showed evidence that intact TetZap70 mice have a specific defect in CD8 lineage development, as TetZap70 CD8s were reduced in frequency in contrast to CD4 SPs that were unaffected (**Chapter 1**, **Figure 1 G**). It has been reported elsewhere that such a CD8 lineage-specific block is exacerbated when TetZap70 cells develop in competition with WT cells in mixed BM chimeras (Saini *et al.*, 2010). A specific defect in DP3 to CD8 SP developmental progression was identified in F5 TetZap70 mice, when they were compared to $F5^{+/-}$ $Rag1^{-/-}$ controls (**Figure 4.4**). This correlated with a reduction in TCR signalling sensitivity following anti-CD3-crosslinking in TetZap70 DP3 cells (**Figure 4.2 A**-**B**). Nevertheless, lower Zap70 expression promoted the phenotypic progression of TetZap70 thymocytes to the DP3 compartment (**Figure 4.1 B**). Taken together, these data suggest that although low levels of Zap70-mediated signal transduction promote DP3 generation, relatively stronger TCR signals may be required for DP3 to CD8 SP developmental progression. Therefore we find evidence that the intrathymic signals required for DP3 to CD8 SP lineage progression are likely to be mediated at least in part through the TCR. Such a conclusion is fitting with a "proof-reading" mechanism for CD8 lineage development (Liu *et al.*, 2003), and contrasts the theory that CD8 lineage development is instructed solely by IL7 and/or gamma-chain (γc) cytokine signals transduced via signal transducer and activator of transcription (STAT)5 and in the absence of TCR signalling, at the $CD4^{hi}CD8^{lo}$ stage (Park *et*

al., 2010; Yu *et al.*, 2003). Furthermore, the presence of CD8 SPs in the IL7, Interleukin-7 receptor α-chain (IL7rα) and γc-deficient mice further argues against a non-redundant instructive role for cytokines in this process (Di Santo *et al.*, 1999; Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995).

We also found evidence that a CD8 lineage-specific developmental block did not simply result from a reduction in Zap70 function. SKG mice exhibit a reduction-of-function mutation in Zap70 (Sakaguchi *et al.*, 2003), which equally affected CD4 and CD8 lineage development (**Figure 4.5**). This fits with the suggestion that the positive selection signal strength correlates with the overall numbers of positively selected cells as opposed to influencing the lineage decision, which researchers have used as evidence to support the kinetic signalling model (Bosselut *et al.*, 2001; Erman *et al.*, 2006). Indeed, a quantitative instructive model predicts that such a loss of Zap70 function and thus reduction in TCR signal, would favour the generation of an increased frequency of CD8 SPs relative to CD4 SPs. In this regard, our results oppose the quantitative instructive model of CD4/CD8 lineage commitment. It could be argued that the phenotypic progression to the DP3 population in TetZap70 mice results from lower Zap70 abundance exacerbating a “break” in the positive selection signal, thus lending support to arguments of the kinetic signalling model (Singer *et al.*, 2008). Therefore our data lends support to aspects of both the quantitative and kinetic models of lineage commitment.

An advance made by our study was to show that during positive selection in WT mice, Zap70 underwent a rapid upregulation in positively selecting subsets of

DP thymocytes (**Figure 4.1 C**), which contributed to increased TCR signalling sensitivity (**Figure 4.2 A**-**B**). It has been shown that Zap70 abundance is higher in thymic SPs relative to total DPs (Chan *et al.*, 1994b; Palacios & Weiss, 2007). However, these studies did not take into account heterogeneity of DP population. As the majority of DP thymocytes are phenotypically DP1 (**Chapter 3**, **Figure 3.4 B**), such studies did not permit resolution of the finer kinetics of Zap70 regulation in developmentally intermediate populations.

Whilst we found that Zap70 was progressively upregulated with maturity during selection (**Figure 4.1 C**), the highest mRNA expression was in the positively selecting DP2 and DP3 populations (**Figure 4.6 A**). This may be necessary as selecting cells rapidly upregulate overall Zap70 expression, whereas expression levels are only required to be maintained in peripheral cells. Furthermore, completion of positive selection has been correlated with the induction of eukaryotic initiation factor 4E (eIF4E), a protein that increases the efficiency of mRNA translation (Nunomura *et al.*, 2000). Therefore Zap70 levels may be maintained in mature SP populations due to increased translation, as opposed to requiring transcriptional elevation. Differences in protein turnover rates in positively selected T-cells compared to non-selecting DPs may also contribute to the maintenance of higher Zap70 abundance.

Interestingly we saw a similar pattern of regulation with regards to CD5 expression. CD5 was rapidly increased in the DP2 population at the transcriptional level, before mRNA expression was reduced in the DP3 and SP populations (**Figure 4.6 B**). This precisely mirrors CD5 protein expression in

the DP populations (**Chapter 1**, **Figure 3.4**). Furthermore, mature SP populations have high levels of CD5 expression, relative to non-selecting DPs (Azzam *et al.*, 1998). This is in agreement with findings suggesting that CD5 is transcriptionally regulated in the thymus by the E protein, E47 (Yang *et al.*, 2004), a factor that is modulated by TCR positive selection signalling (reviewed in (Murre, 2005)). However, our finding that CD5 is downregulated at the DP3 stage suggests a further level of developmental regulation. We found that WT DP3 cells were actually more sensitive to TCR signals compared to DP2s, suggesting they should indeed have higher levels of CD5, if expression was entirely regulated by TCR signalling (**Figure 4.2 A**). An intriguing possibility is that the developmental tuning of CD5 contributes to the increasing TCR signalling sensitivity of DP3 cells, allowing interpretation of inherently weaker MHC-I mediated signals. Further experiments will be required to elucidate whether developmental modulation of E proteins directly contributes to the regulation of CD5 expression in DP3 thymocytes.

Nevertheless, because both Zap70 and CD5 showed similar expression patterns (with the exception of the DP3 population), and due to the implication of TCR signalling in CD5 regulation (Azzam *et al.*, 1998), the evidence was certainly suggestive of a direct role for TCR signalling in Zap70 modulation. To further explore this possibility, we undertook a comparative genomics approach to identify non-coding ECRs, which may correlate with genetic regulatory elements. We found a number of conserved binding sites for transcription factors which themselves are direct targets of TCR signalling, including an Ets,

Ebox, NFAT and CREB site (**Figure 4.7**). Furthermore, we showed that PMA and ionomycin treatment at a concentration known to mimic the positive selection signal, also upregulated Zap70 (**Figure 4.8 B-C**) (Ohoka *et al.*, 1996). In support of the finding that TCR signalling may directly upregulate Zap70, Chandok et al. recently found that Zap70 is upregulated following antigenic stimulation of peripheral T-cells (Chandok *et al.*, 2007). We additionally showed that the calcineurin inhibitors CsA and FK506 inhibited this upregulation suggesting that NFAT activation may be particularly important for this process (**Figure 4.8 F-H**). There is considerable redundancy between NFAT proteins, as NFAT1, NFAT2 and NFAT4 are expressed in thymocytes (reviewed in (Macian, 2005)), and their expression is temporally regulated by the positive selection signal (Adachi *et al.*, 2000). NFAT4 predominates in DP thymocytes, and interestingly the *Nfat4*$^{-/-}$ mouse has an apparently greater defect in the CD8 lineage development relative to the CD4 lineage, although this was not quantified in a large cohort of mice (Oukka *et al.*, 1998). If this proved to be the case, it is an interesting possibility that this reflects a failure to upregulate Zap70 during positive selection in these mice. Further analysis of the *Nfat4*$^{-/-}$ mice would be required determine if Zap70 was indeed dysregulated during positive selection. In addition, it will be important in future experiments to determine whether NFAT directly associates with the *Zap70* gene. ChIP analysis could be employed to determine whether NFAT binds to the *Zap70* locus directly. Furthermore, generation of mice with a mutation of the conserved NFAT binding site will enable elucidation of the precise role of this site in *Zap70* gene regulation.

A criticism of the finding that PMA and ionomycin treatment can induce Zap70 expression in DPs, is that whilst this treatment is known to mimic positive selection TCR signalling, it certainly deviates from the physiological signal. We therefore undertook studies *in vivo*, aimed at investigating the kinetics of Zap70 upregulation. First of all we found that I.P. injection of anti-CD3/CD28 induced an upregulation of Zap70 in some F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ cells (**Figure 4.9 A**). Secondly, we showed that F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ cells received a stronger positive selection signal following intrathymic transfer to B6 hosts relative to F5 *Rag1*$^{-/-}$ hosts, likely resulting from differences in intraclonal competition between the host environments. Importantly, we found that increased kinetics of Zap70 upregulation in donor F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes correlated with stronger positive selection signalling (**Figure 4.9**). Thus we conclude that the positive selection TCR signal is likely to directly upregulate Zap70 expression. Furthermore, this suggests there is a reciprocal relationship between TCR signalling and Zap70, as Zap70 expression is also rate-limiting for TCR signal transduction (**Figure 4.1 A**). Intriguingly, the finding that the strength of the TCR signal dictates the extent to which Zap70 is upregulated may have implications on the future homeostatic potential of a selecting cell. For example, cells with a relatively weak avidity TCR may not fully increase their Zap70 expression levels. This may impact the antigen sensitivity of a T-cell clone in the periphery. Furthermore, because TCR signalling is necessary for naïve T-cell survival (Ernst *et al.*, 1999; Polic *et al.*, 2001; Viret *et al.*, 1999), the lifespan of a low avidity T-cell may be impacted. How the positive selection

signal impacts on the homeostatic potential of a T-cell is investigated further in **Chapter 5**.

Whilst we find evidence that TCR signals positively upregulate Zap70, Zap70 itself is likely to be a rate-limiting in TCR signal transduction. By increasing Zap70 expression, the TCR signal may induce a positive feedback loop, which further increases TCR signalling sensitivity. Such a model is fitting with a feedforward-loop, which is an evolutionary conserved signalling network motif, commonly involved in discerning instructive developmental signals over background noise (reviewed in (Alon, 2007)). We suggest that signals through the TCR augment TCR sensitivity. This increased intracellular sensitivity in conjunction with consistent extracellular TCR signalling instructs the lineage commitment process. With regards to the lineage decision itself, it is known that CD4 has a higher affinity for Lck than does CD8, implying that CD4-mediated signals are relatively stronger than those transduced by CD8 (Ravichandran & Burakoff, 1994; Wiest *et al.*, 1993). We would therefore predict that MHC-II restricted T-cells will exhibit a more rapid upregulation of Zap70 compared to MHC-I restricted cells. Such a possibility will require further dissection, by following Zap70 expression in positively selecting DP1 thymocytes, following intrathymic transfer to *b2m*$^{-/-}$ or MHC-II Δ/Δ hosts.

CD4 lineage cells develop with earlier timings than CD8 lineage cells (**Chapter 1**). Therefore, strong/consistent MHC-II-mediated signals could be resolved over weak/intermittent MHC-I-mediated signals through differential kinetics of TCR signalling sensitisation. Alternatively, because CD4-mediated

signals are of a higher strength relative to those mediated by CD8, positive selection signal transduction may occur in MHC-II restricted cells when Zap70 is more limiting. Conceivably this may occur independently of Zap70 upregulation for MHC-II-restricted cells in the DP2 population. In contrast, positive upregulation of TCR signalling strength may be needed to discern weaker/intermittent signals, a prediction not made by the classical quantitative instructive model. With regards to the kinetic signalling model, evidence suggests that the $CD4^{hi}CD8^{lo}$ coreceptor reversing population correlates with increased signalling strength (Correia-Neves *et al.*, 2001; Ohashi *et al.*, 1993; Singer *et al.*, 2008) (reviewed in (Hogquist, 2001)). It could be that coreceptor reversal may simply represent a mechanism to decrease the early signal received by high avidity MHC-I restricted cells (Correia-Neves *et al.*, 2001) (reviewed in (He *et al.*, 2010)). Re-expression of the CD8 coreceptor would then permit strong signalling to occur in DP3 cells. Thus the quantitative and kinetic models of thymic development may not be mutually exclusive, however the changes in TCR signalling threshold and kinetic aspects of CD4/CD8 lineage commitment are crucial to the lineage commitment process.

Thus we conclude with the following model for the signalling requirements of positive selection and the CD4/CD8 lineage decision (**Figure 4.10**). We suggest that upon transit to the DP2 population, MHC-II cells receive a relatively stronger and more consistent TCR signal, permitting rapid upregulation of Zap70, in conjunction with lineage commitment. In contrast, because DP2 cells are relatively insensitive to TCR signalling, we suggest that weakly/intermittently

signalled MHC-I restricted cells are refractive to lineage specifying signals at this stage. However, a lower level of signalling permits the upregulation of Zap70 and the phenotypic transition to the DP3 population. DP3 cells are more sensitive to receiving TCR signals, and thus this may permit the interpretation of MHC-I-mediated TCR signals at this later stage. Therefore both quantitative and kinetic aspects contribute to the CD4 versus CD8 lineage decision. An essential feature of such a model is that temporal separation of signals through the same receptor can lead to divergent developmental fates. The temporal separation of developmental cues is consistent with a permissive signalling threshold model, a conserved developmental mechanism whereby gradients in receptor sensitivity permit resolution of temporally distinct signals (reviewed in (Freeman & Gurdon, 2002)). A future goal for developmental biologists will be the identification of factors that switch the amenability of a cell between different potential fates. Microarray analysis of the DP2 versus DP3 populations may facilliate the identification of such factors in positively selecting thymic populations.

Figure 4.1 Transgenic Zap70 expression is rate-limiting for TCR signal transduction and can influence the developmental fate of thymocytes

(**A**) TetZap70 mice were fed dox for two days (red line) and were analysed by flow cytometry, along with *Zap70*$^{-/-}$ (grey fill) controls. The left histogram shows the expression of HuCD2 on live thymocytes from TetZap70 and *Zap70*$^{-/-}$ mice. Cells were electronically gated based on expression of HuCD2 (1-5, gate 5 refers to total *Zap70*$^{-/-}$ control thymocytes). The right hand histogram shows the percentage of thymocytes from gates 1-5 influxing calcium, as a function of time. Addition of CD3-crosslinking antibody is denoted as X. (**B**-**C**) TetZap70^{on}, B6 and *Zap70*$^{-/-}$ (grey fill) mice were analysed by flow cytometry, for the expression of Zap70. (**B**) The top histogram shows the expression of Zap70 in *Zap70*$^{-/-}$ controls (grey fill), in addition to HuCD2^{+} gated DP1 cells (black line), DP2 cells (red line) and DP3 cells (blue line), from TetZap70^{on} mice. The bottom histogram shows the expression of Zap70 in *Zap70*$^{-/-}$ controls (grey fill) in addition to TCR^{+}CD5^{+} CD4 SP (red line) and TCR^{+}CD5^{+} CD8 SP cells (blue line), from TetZap70^{on} mice. Zap70 mean fluorescence intensity (MFI) values are for indicated populations are shown next to histograms. (**C**) The top histogram shows the expression of Zap70 in *Zap70*$^{-/-}$ controls (grey fill), in addition to DP1 cells (black line), DP2 cells (red line) and DP3 cells (blue line), from B6 mice. The bottom histogram shows the expression of Zap70 in *Zap70*$^{-/-}$ controls (grey fill) in addition to TCR^{+}CD5^{+} CD4 SP (red line) and TCR^{+}CD5^{+} CD8 SP cells (blue line), from B6 mice. Zap70 MFI values are for indicated populations are shown next to histograms. Data in (**A**-**C**) is representative of ≥3 independent experiments. (**D**) DP1-3, CD4 SP and CD8 SP populations were

sorted to high purity (≥95%) from B6 mice. Cell populations were lysed and protein equivalent to $2x10^5$ cells was run on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was stained for Zap70 (top) or GAPDH (bottom). Antibody staining was detected by secondary incubation with a horseradish peroxidase (HRP)-coupled secondary antibody and resolution of chemiluminescence following treatment with enhanced chemiluminescence (ECL) reagent. Zap70 expression was normalised to GAPDH based on the relative chemiluminescence. Data is representative of two independent experiments.

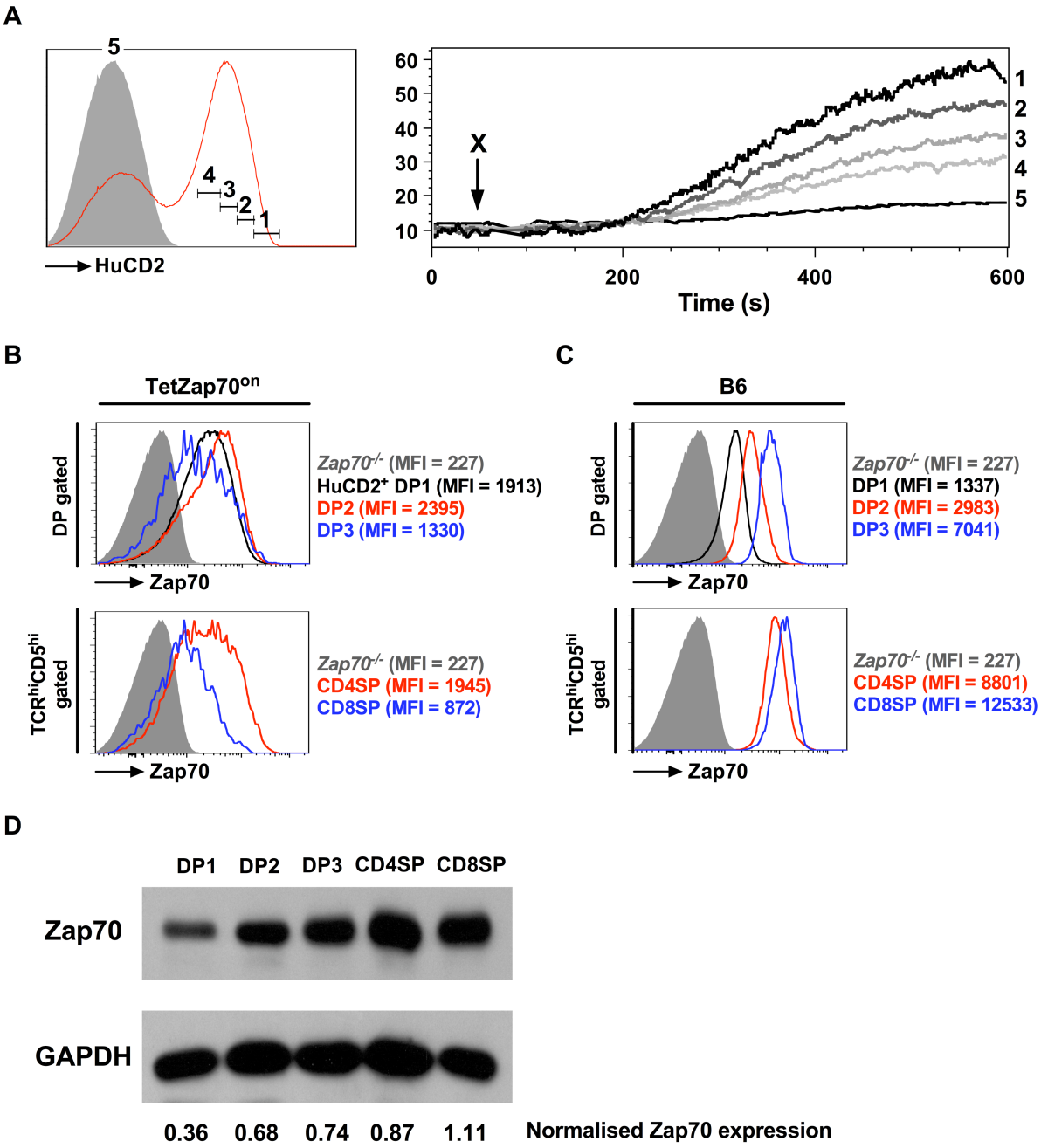

A
5
4
3
2
1
HuCD2
60
50
40
30
20
10
X
1
2
3
4
5
0
200
400
600
Time (s)
B
TetZap70on
DP gated
Zap70-/- (MFI = 227)
HuCD2+ DP1 (MFI = 1913)
DP2 (MFI = 2395)
DP3 (MFI = 1330)
Zap70
TCRhiCD5hi gated
Zap70-/- (MFI = 227)
CD4SP (MFI = 1945)
CD8SP (MFI = 872)
Zap70
C
B6
DP gated
Zap70-/- (MFI = 227)
DP1 (MFI = 1337)
DP2 (MFI = 2983)
DP3 (MFI = 7041)
Zap70
TCRhiCD5hi gated
Zap70-/- (MFI = 227)
CD4SP (MFI = 8801)
CD8SP (MFI = 12533)
Zap70
D
DP1 DP2 DP3 CD4SP CD8SP
Zap70
GAPDH
0.36 0.68 0.74 0.87 1.11
Normalised Zap70 expression

Figure 4.2 Defective TCR signalling in TetZap70 DP3 thymocytes following CD3 crosslinking

TetZap70 mice were fed dox for ≥4 days and were analysed for the expression of CD4, CD8, TCR and CD5 by flow cytometry, along with B6 controls. (**A**-**B**) Histograms show the percentage of DP1 (thin black line), DP2 (red line), DP3 (blue line) and B6 CD8 SP (thick black line) thymocytes influxing calcium as a function of time. Addition of CD3-crosslinking antibody is denoted as X. Histograms represent DP populations from (**A**) B6 and (**B**) TetZap70 mice. Data is representative of ≥3 independent experiments. (**C**) DP1 (top row), DP2 (middle row) and DP3 (bottom row) thymocytes from TetZap70 (red line) and B6 (black line) mice were analysed by flow cytometry. Left histograms show the percentage thymocytes influxing calcium as a function of time, following addition of ionomycin (denoted as Iono). Right histograms show Indo1 expression following loading and prior to the induction of calcium influx.

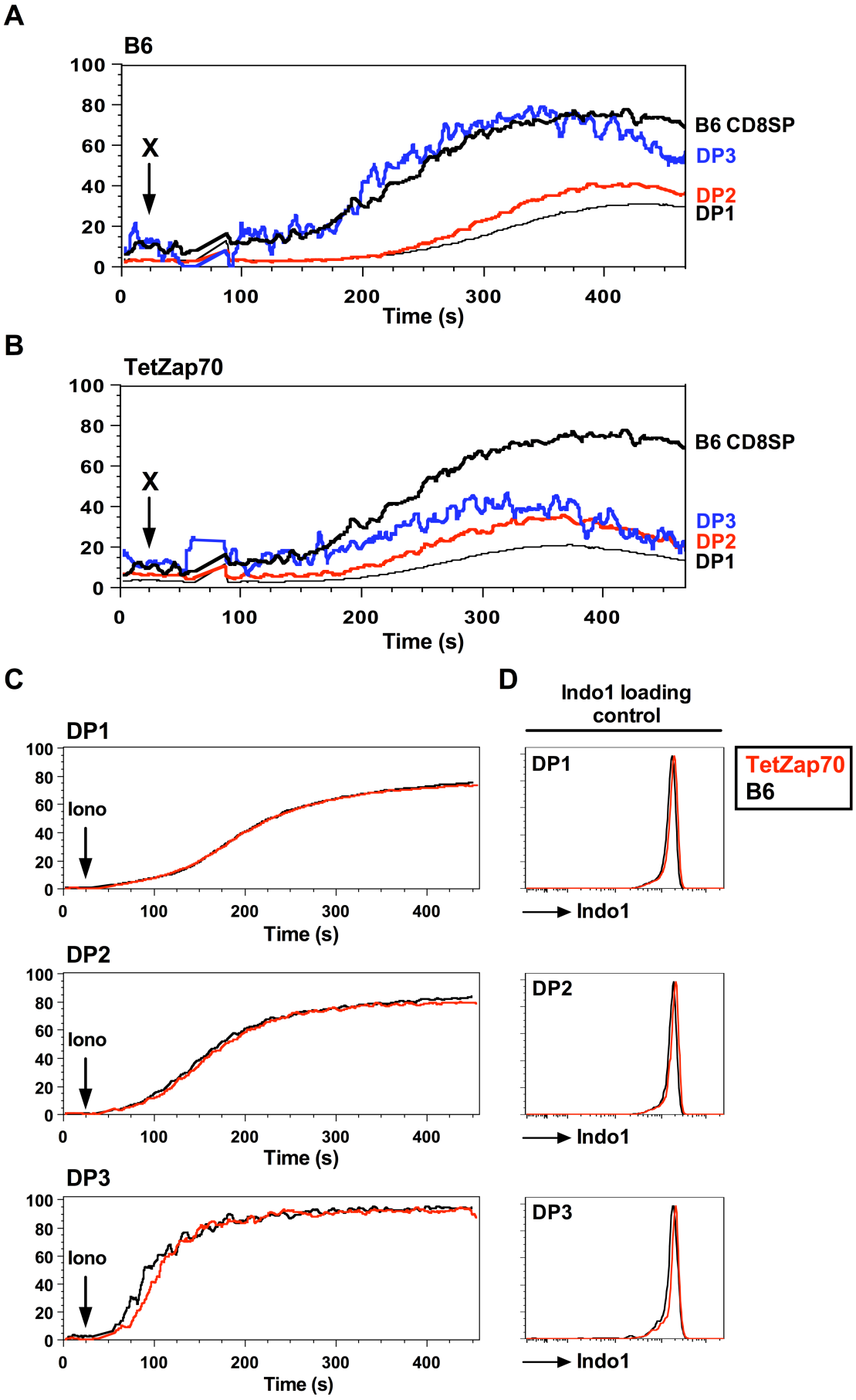

A
B6
100
80
60
40
20
0
X
0
100
200
300
400
Time (s)
B6 CD8SP
DP3
DP2
DP1
B
TetZap70
100
80
60
40
20
0
X
0
100
200
300
400
Time (s)
B6 CD8SP
DP3
DP2
DP1
C
DP1
100
80
60
40
20
0
Iono
0
100
200
300
400
Time (s)
DP2
100
80
60
40
20
0
Iono
0
100
200
300
400
Time (s)
DP3
100
80
60
40
20
0
Iono
0
100
200
300
400
Time (s)
D
Indo1 loading control
DP1
Indo1
DP2
Indo1
DP3
Indo1
TetZap70
B6

Figure 4.3 TCR signal-transducing proteins are not dysregulated in TetZap70 mice

TetZap70 mice (red line) were fed dox for ≥4 days and were analysed by flow cytometry, along with B6 controls (black line). DP1 DP2 and DP3 populations were discriminated by staining for CD4 CD8 TCR and CD5 and were further analysed for the expression of intracellular Lck, Fyn, LAT, Slp76 or Erk2. Expression levels were compared to the following negative controls: Lck expression was compared to *Lck*$^{-/-}$ DP1 cells, Fyn and Erk2 expression were compared to isotype control stained B6 DP1 cells and LAT and Slp76 expression were compared to B6 TCR^{-}CD5^{-}CD25^{-}CD44^{-} lymphocytes, comprising primarily of B-cells. Results are representative of ≥3 independent experiments.

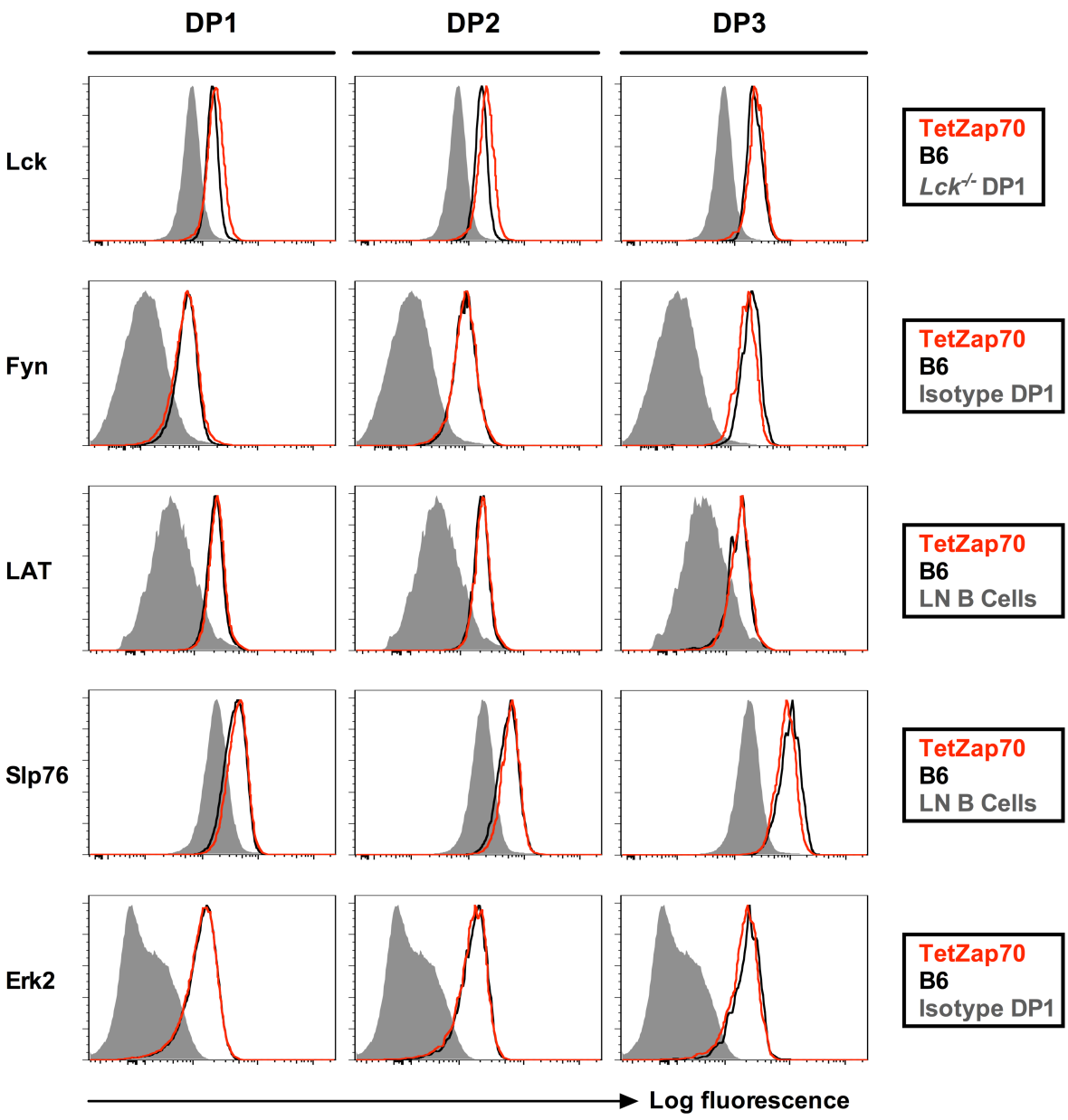
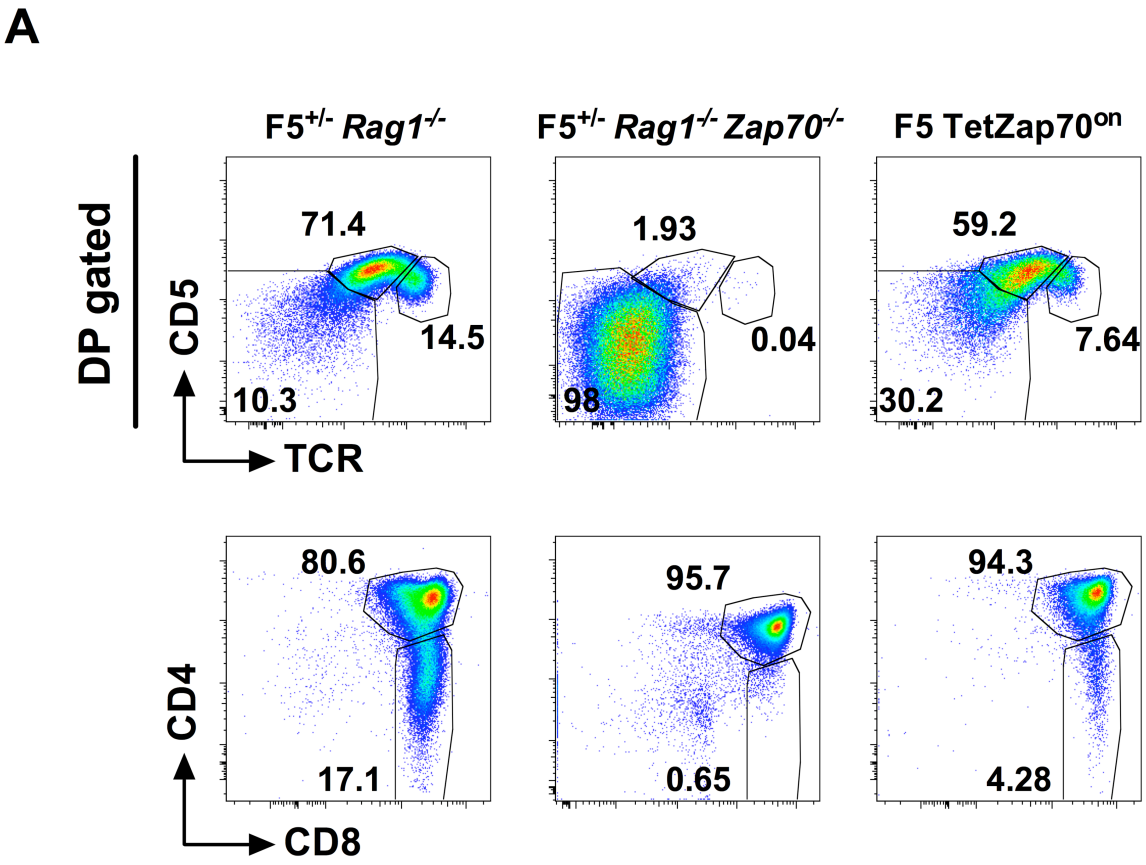

DP1
DP2
DP3
Lck
Fyn
LAT
Slp76
Erk2
TetZap70
B6
Lck-/- DP1
TetZap70
B6
Isotype DP1
TetZap70
B6
LN B Cells
TetZap70
B6
LN B Cells
TetZap70
B6
Isotype DP1
Log fluorescence

Figure 4.4 A developmental block between DP3 and CD8 SP populations is a consequence of transgenic Zap70 expression

F5 TetZap70 mice were fed dox for ≥7 days (F5 TetZap70^{on}) and were analysed for the expression of CD4, CD8, TCR, CD5 and HuCD2 by flow cytometry, along with F5$^{+/-}$ *Rag1*$^{-/-}$ and F5$^{+/-}$ *Rag1*$^{-/-}$ *Zap70*$^{-/-}$ controls. (**A**) Density plots show expression of CD5 against TCR on DP gated thymocytes and CD4 against CD8 on live gated thymocytes (DP gating shown on bottom row, F5 TetZap70^{on} cells are additionally HuCD2^{+} gated). (**B**) Bar charts show the DP3:DP2 and CD8:DP3 ratio in F5$^{+/-}$ *Rag1*$^{-/-}$ controls (n=2) and F5 TetZap70^{on} mice (n=3). DP3:DP2: p=ns and CD8:DP3: ** p≤0.01 (Student's t-test).


A
F5+/- Rag1-/-
F5+/- Rag1-/- Zap70-/-
F5 TetZap70on
DP gated
CD5
TCR
71.4
14.5
10.3
1.93
0.04
98
59.2
7.64
30.2
CD4
CD8
80.6
17.1
95.7
0.65
94.3
4.28

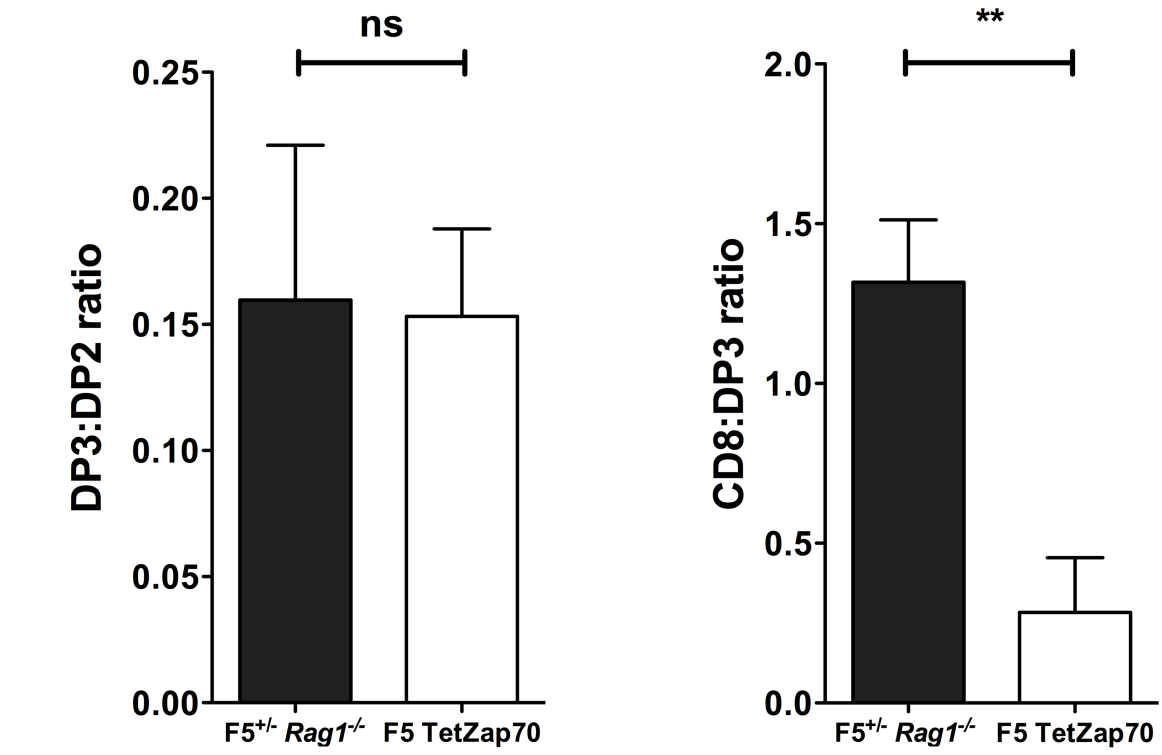

B
ns
DP3:DP2 ratio
0.25
0.20
0.15
0.10
0.05
0.00
F5+/- Rag1-/-
F5 TetZap70
**
CD8:DP3 ratio
2.0
1.5
1.0
0.5
0.0
F5+/- Rag1-/-
F5 TetZap70

Figure 4.5 Reduced Zap70 function affects CD4 and CD8 lineages equally

SKG mice and BALB/c control thymi and LNs were analysed for the expression of CD4, CD8, CD5, TCR and Zap70 by flow cytometry. (**A**) Scatter dot plot shows total live thymocyte numbers. p=ns (Student's t-test BALB/c n=10, SKG n=10). (**B**) Scatter dot plot shows the percentage of $TCR^{hi}CD5^{hi}$ cells as a frequency of live thymocytes. *** p≤0.001 (Student's t-test BALB/c n=10, SKG n=10). (**C**) Density plots show expression of CD4 against CD8 on live thymocytes (top) and CD5 against TCR (bottom) on DP gated thymocytes. (**D**) Scatter dot plot shows total live $TCR^{+}CD5^{+}$ LN cells. *** p≤0.001 (Student's t-test BALB/c n=10, SKG n=10). (**E**) Density plots show CD4 against CD8 on $CD25^{-}CD44^{lo}$ (naïve) LN lymphocytes. (**F**) Bar charts show the CD4:CD8 ratio of $TCR^{hi}CD5^{hi}$ mature thymocytes (p=ns, Student's t-test BALB/c n=10, SKG n=10) and naïve LN lymphocytes (p=ns, Students t-test BALB/c n=5, SKG n=5). (**G**) Histograms show Zap70 expression in DP1 (grey fill), DP2 (top row, red line), DP3 (top row, blue line), CD4 SP (bottom row, red line) and CD8 SP (bottom row, blue line) populations. MFI values are indicated to the right of histograms. Data is representative of ≥3 independent experiments.

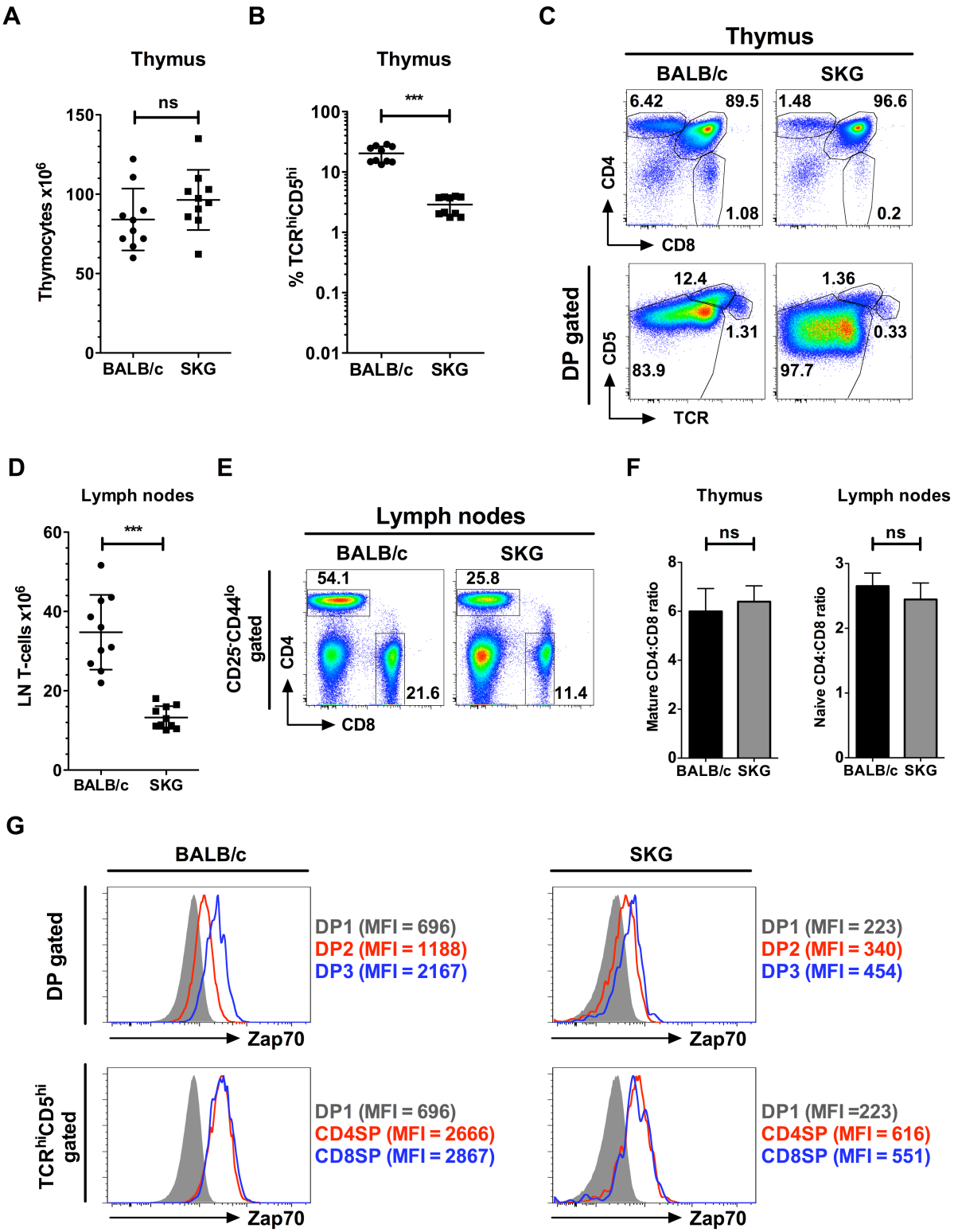

A
Thymus
ns
Thymocytes x10^6
150
100
50
0
BALB/c
SKG
B
Thymus
***
% TCR^hiCD5^hi
100
10
1
0.1
0.01
BALB/c
SKG
C
Thymus
BALB/c
SKG
6.42
89.5
1.08
1.48
96.6
0.2
CD4
CD8
DP gated
12.4
1.31
83.9
1.36
0.33
97.7
CD5
TCR
D
Lymph nodes
***
LN T-cells x10^6
60
40
20
0
BALB/c
SKG
E
Lymph nodes
BALB/c
SKG
CD25-CD44lo gated
54.1
21.6
25.8
11.4
CD4
CD8
F
Thymus
ns
Mature CD4:CD8 ratio
8
6
4
2
0
BALB/c
SKG
Lymph nodes
ns
Naive CD4:CD8 ratio
3
2
1
0
BALB/c
SKG
G
BALB/c
SKG
DP gated
DP1 (MFI = 696)
DP2 (MFI = 1188)
DP3 (MFI = 2167)
Zap70
DP1 (MFI = 223)
DP2 (MFI = 340)
DP3 (MFI = 454)
Zap70
TCR^hiCD5^hi gated
DP1 (MFI = 696)
CD4SP (MFI = 2666)
CD8SP (MFI = 2867)
Zap70
DP1 (MFI =223)
CD4SP (MFI = 616)
CD8SP (MFI = 551)
Zap70

Figure 4.6 Zap70 and CD5 are transcriptionally regulated during positive selection

DP1, DP2, DP3, CD4 SP and CD8 SP populations were sorted to high purity (≥95%) from B6 mice. Cellular mRNA was purified and analysed by RT-qPCR. Bar charts show the mean expression of (**A**) Zap70 and (**B**) CD5 relative to Hprt1. Analysis was performed in triplicate wells and error bars represent the sd. Data is representative of ≥3 independent experiments.

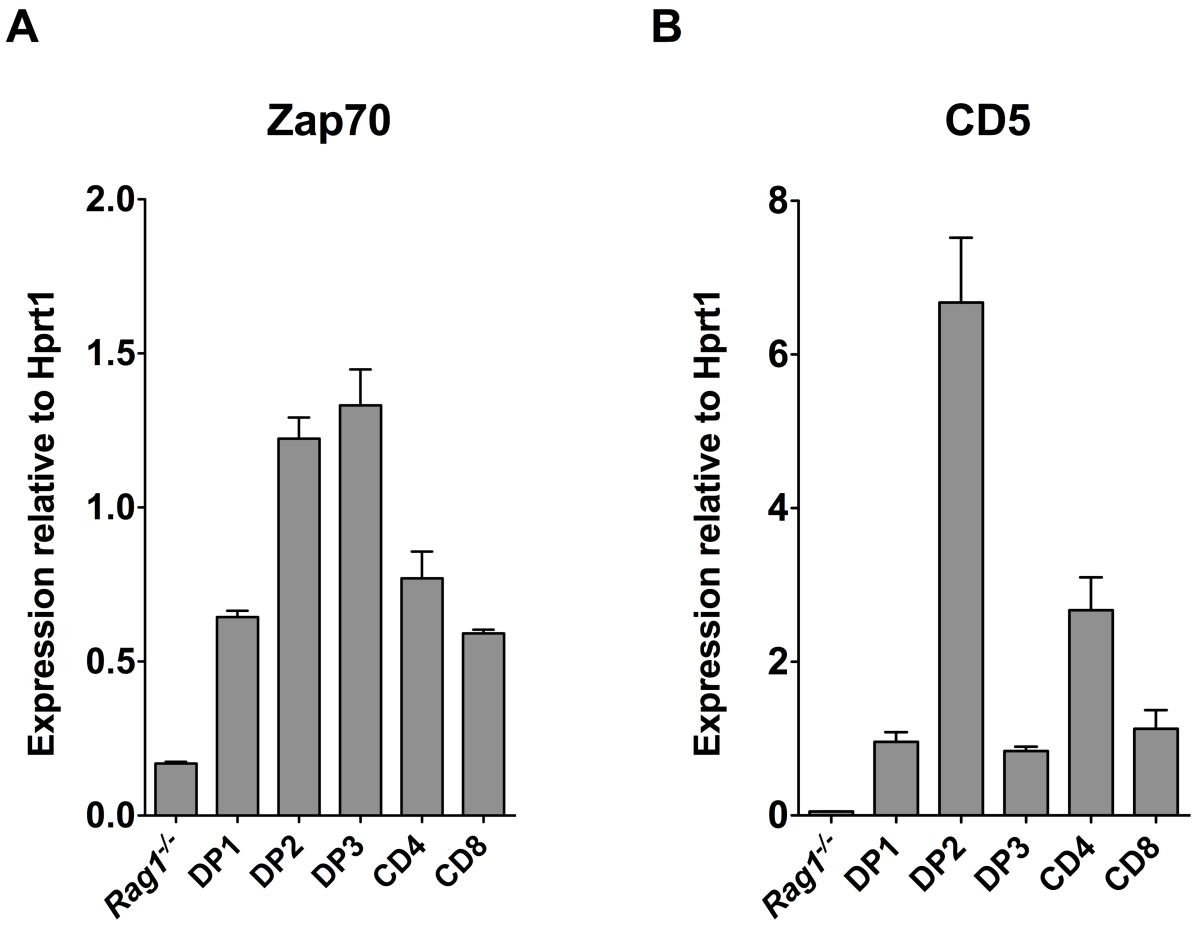
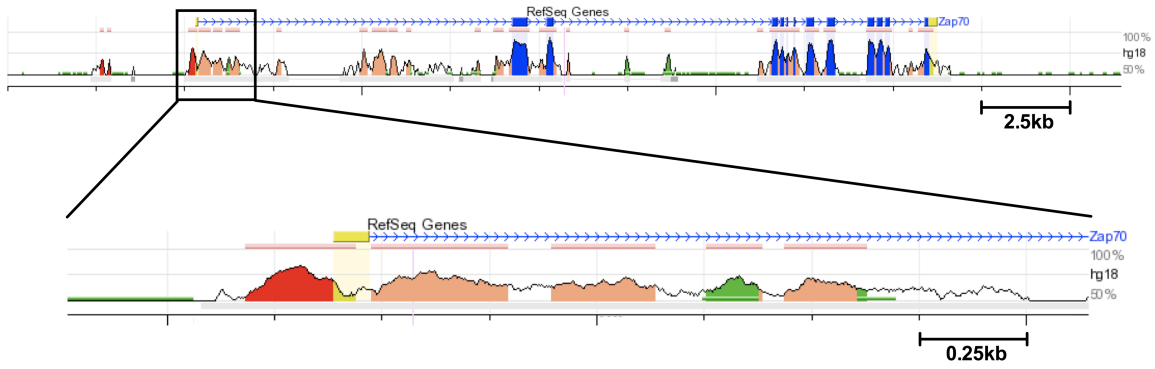

A
Zap70
Expression relative to Hprt1
2.0
1.5
1.0
0.5
0.0
Rag1-/-
DP1
DP2
DP3
CD4
CD8
B
CD5
Expression relative to Hprt1
8
6
4
2
0
Rag1-/-
DP1
DP2
DP3
CD4
CD8

Figure 4.7 Evolutionary conserved transcription factor binding analysis of the region upstream of *Zap70* identifies potential regulatory factors

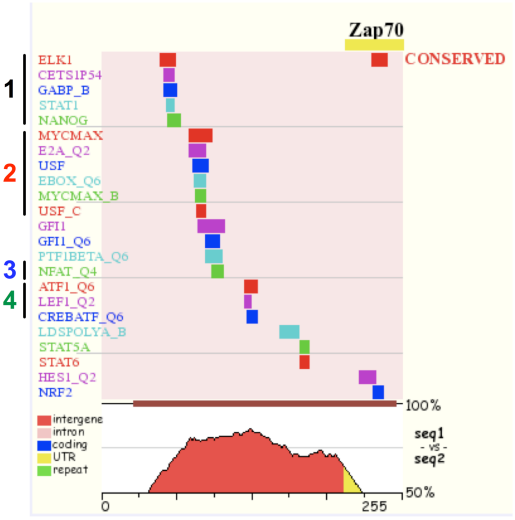
Comparative genomic analysis was performed on murine and human DNA encoding *Zap70* and ~5-10 kb proximal regions using the ECR browser (available online at http://ecrbrowser.dcode.org/, accessed September 2010). (**A**) Graphical output showing evolutionary conserved regions (coloured histograms) between human and mouse DNA. The 5' putative *Zap70* promotor is shown magnified. Histograms represent evolutionary conserved non-coding regions (red), transposons and simple repeats (green), intronic regions (salmon), coding exons (blue) and untranslated regions (yellow). (**B**) Left hand graphical output shows evolutionary conserved transcription factor binding sites as identified by rVista 2.0 using the TRANSFAC professional V10.2 library for vertebrates, with matrix similarity optimised for function. The right panel shows the mouse (top) versus human (bottom) DNA alignment. Ets (1, black), Ebox (2, red), NFAT (3, blue) and CREB (4, green) evolutionary conserved transcription factor binding sites are indicated on the left graphical output (coloured numbers) and the right hand DNA alignment (coloured boxes).

A



B

Evolutionary conserved TF binding sites



1 - Ets
2 - Ebox
3 - NFAT
4 - CREB

Mouse vs human alignment

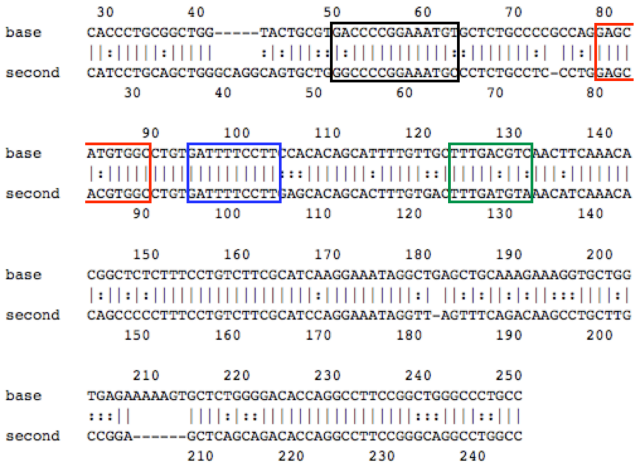


Figure 4.8 Mimicking the positive selection signal upregulates Zap70 *in vitro*

B6 thymocytes were depleted of $CD3^+$, $CD25^+$ and $CD69^+$ cells using magnetic beads. Thymocytes ($5x10^6$ cells/mL) were cultured with PMA (P) and ionomycin (I) for 14 hours, before being washed and subsequently cultured in medium alone for the remaining culture time. Where indicated, inhibitors were added for the entire duration of the culture. Non-treated cells (NT, grey fill), PMA and ionomycin treated cells (P+I, black line) or PMA, ionomycin and inhibitor treated cells were washed and subsequently analysed for the expression of CD4, CD8, TCR, CD5, CD69 and Zap70 by flow cytometry. (**A**) Density plots show expression of CD4 against CD8 and CD5 against TCR on DP gated cells, on pre- and post-depletion thymocytes. (**B**) Cells were cultured with different combinations of PMA and/or ionomycin. Density plots show the expression of CD4 against CD8 at 22 and 44 hours. (**C**) Histograms show the expression of CD69 and Zap70 in Non-treated cells, cells treated with PMA alone (red line), ionomycin alone (blue line) or PMA and ionomycin after 22 and 44 hours. (**D**-**H**) Cells were analysed after 22 hours. (**D**) Cells were cultured in medium alone, with PMA and ionomycin, or with PMA, ionomycin and actinomycin D (ActD, red line) or cyclohexamide (Chx, blue line). Density plots show CD4 against CD8 expression on live thymocytes, and the histogram shows Zap70 expression in live thymocytes. (**E**) Thymocytes were cultured in medium alone, with PMA and ionomycin, or with PMA, ionomycin and Kn93 (red line) or U0126 (blue line). The histogram shows Zap70 expression in live thymocytes. (**F-G**) Cells were cultured with PMA and ionomycin along with

different concentrations of cyclosporin A (CsA). (**F**) Density plots show CD4 against CD8 in live thymocytes. (**G**) Histogram shows Zap70 expression on live thymocytes. (**H**) Histogram shows Zap70 on live thymocytes cultured in medium, with PMA and ionomycin or with PMA, ionomycin and FK506 (red line). Data is representative of ≥2 independent experiments.

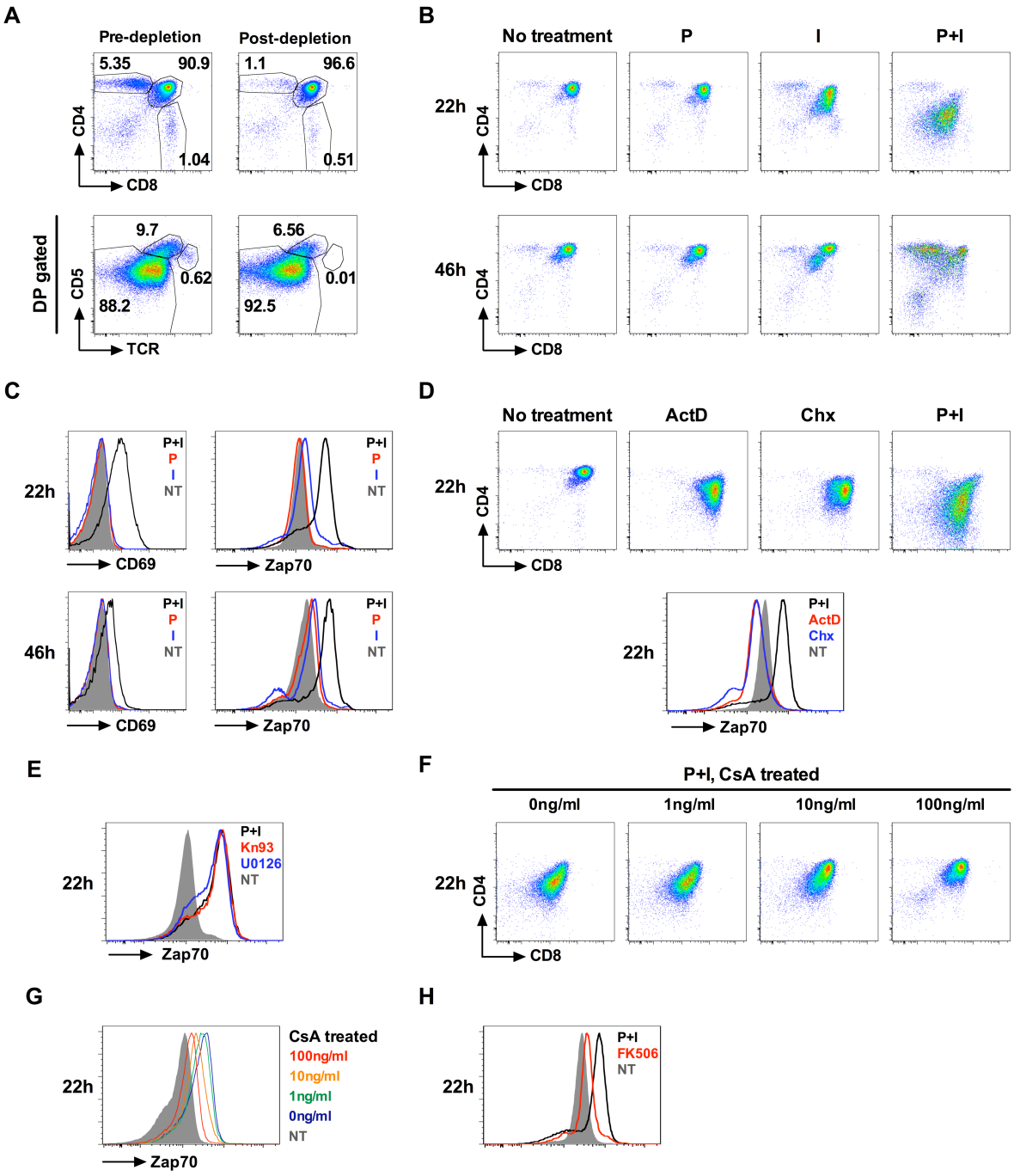

A
Pre-depletion
Post-depletion
5.35
90.9
1.04
1.1
96.6
0.51
CD4
CD8
DP gated
9.7
0.62
88.2
6.56
0.01
92.5
CD5
TCR
B
No treatment
P
I
P+I
22h
46h
CD4
CD8
C
22h
46h
P+I
P
I
NT
CD69
Zap70
D
No treatment
ActD
Chx
P+I
22h
CD4
CD8
P+I
ActD
Chx
NT
Zap70
E
22h
P+I
Kn93
U0126
NT
Zap70
F
P+I, CsA treated
0ng/ml
1ng/ml
10ng/ml
100ng/ml
22h
CD4
CD8
G
22h
CsA treated
100ng/ml
10ng/ml
1ng/ml
0ng/ml
NT
Zap70
H
22h
P+I
FK506
NT

Figure 4.9 Zap70 is regulated by the positive selection TCR signal

(**A**-**C**) F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ mice were injected I.P. with 50*μ*g anti-CD3 (clone H57-597, eBioscience) and 5*μ*g anti-CD28 (clone 37.51, eBioscience) and analysed for the expression of CD4, CD8, TCR and CD5 by flow cytometry after 24 hours, along with non-injected controls. (**A**) Density plots show CD4 against CD8 on live thymocytes and CD5 against TCR on DP gated thymocytes (DP gating shown on the top row plots). (**B-C**) DP1 (black line) and DP2 (red line) populations from anti-CD3 and anti-CD28 treated mice and total DPs from non-injected mice (grey fill) were analysed for the expression of CD69 and Zap70. Histograms show (**B**) CD69 and (**C**) Zap70 expression. Data represents two independent experiments. (**D**-**E**) F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} thymocytes (1-2x10^{6} cells) were intrathymically transferred to B6 CD45.1^{+} hosts (red line) or F5 *Rag1*$^{-/-}$ CD45.1^{+} hosts (blue line). Host and donor thymocytes were analysed by flow cytometry 1-5 days following transfer. (**D**) Histogram shows CD69 expression on live CD45.2^{+} donor thymocytes or B6 DP1 host (CD45.1^{+}) controls (grey fill). (**E**) Line graph shows the fold increase of Zap70 MFI in donor cells (CD45.2^{+}) relative to the host (CD45.1^{+}) mouse DP1 population. The fold increase in Zap70 MFI in CD8 SPs relative to the host DP1 population is also shown for F5 *Rag1*$^{-/-}$ CD45.1^{+} (black circle) and B6 CD45.1^{+} (black square) host thymocytes. Data represents ≥2 transfers per timepoint.

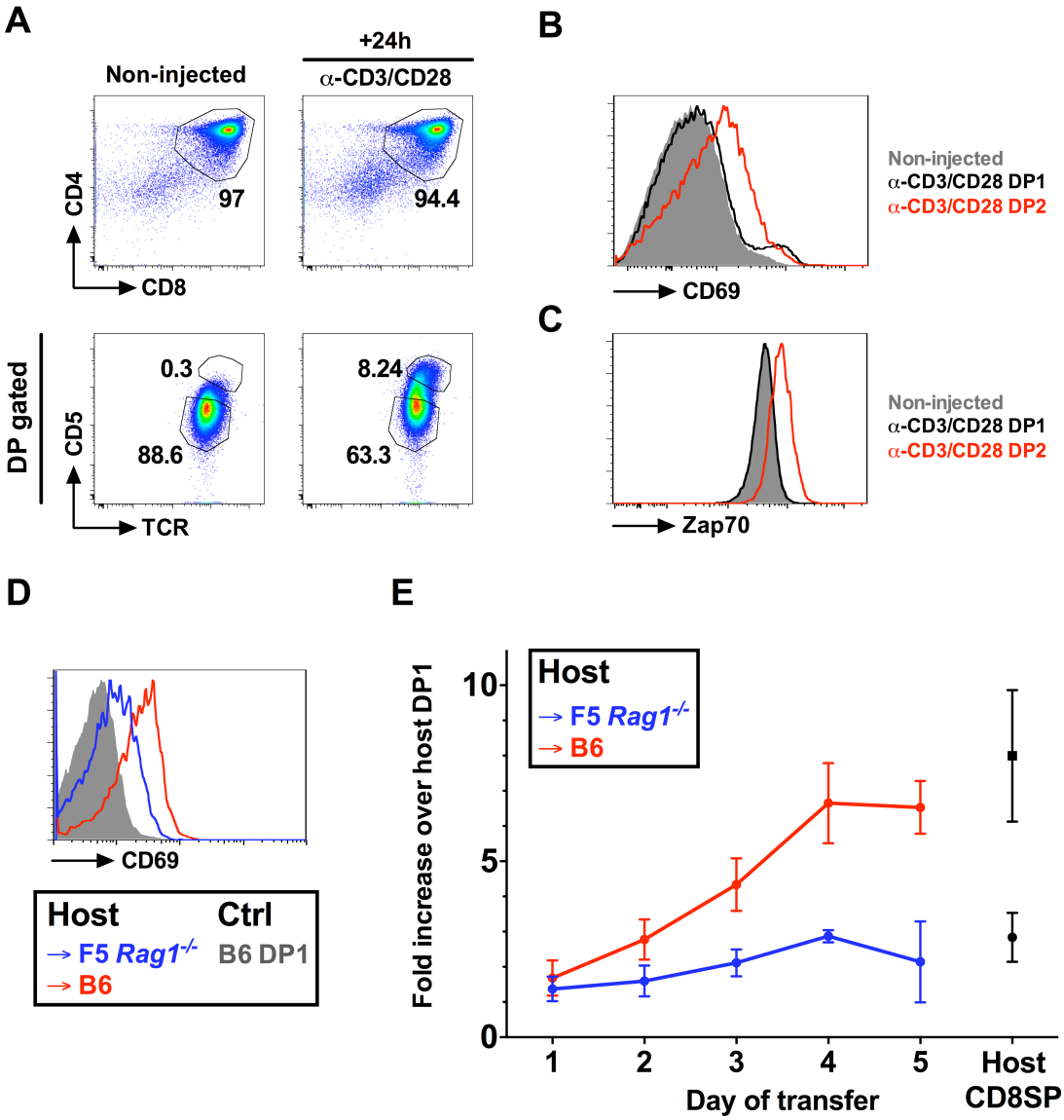
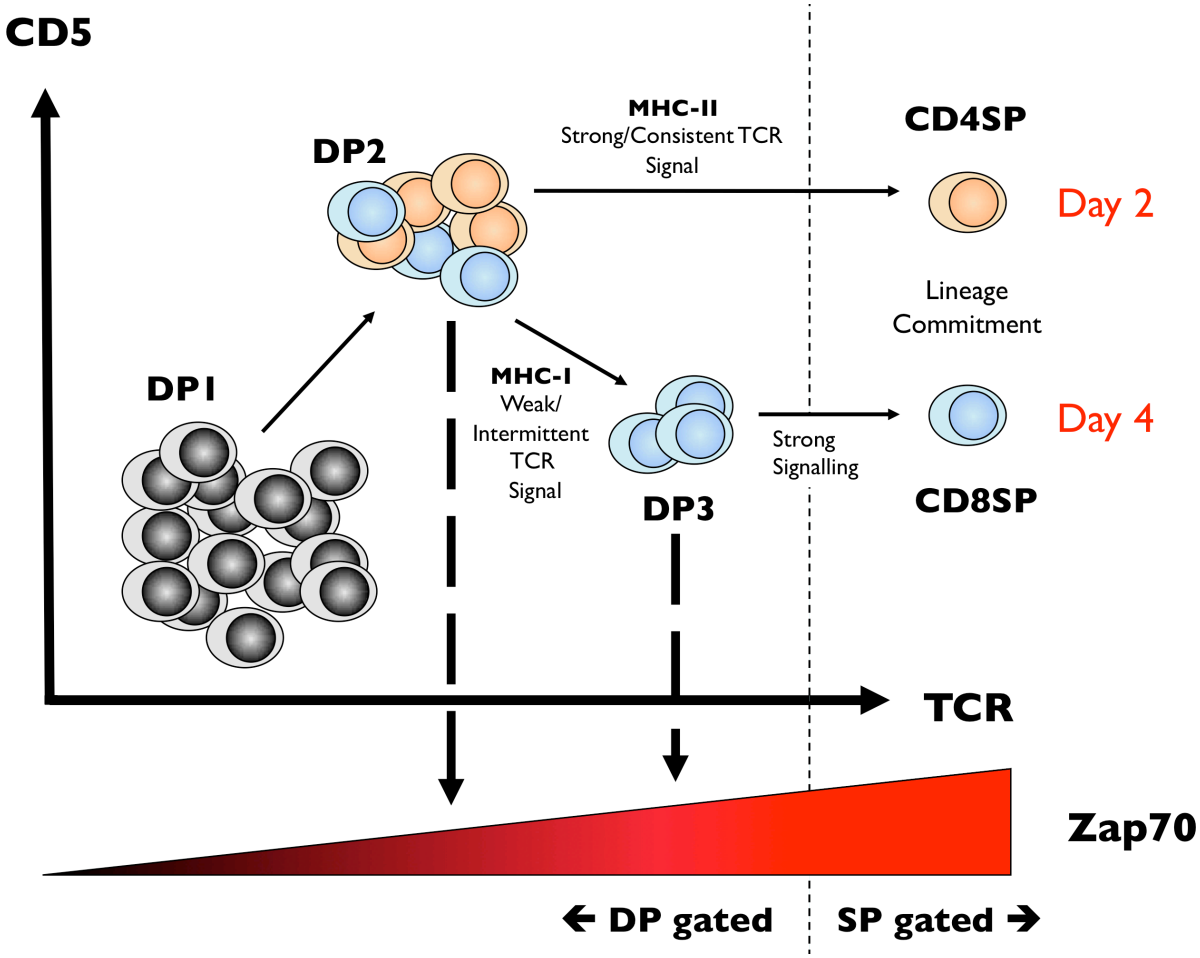

A
Non-injected
+24h
α-CD3/CD28
CD4
CD8
97
94.4
DP gated
CD5
TCR
0.3
88.6
8.24
63.3
B
CD69
Non-injected
α-CD3/CD28 DP1
α-CD3/CD28 DP2
C
Zap70
Non-injected
α-CD3/CD28 DP1
α-CD3/CD28 DP2
D
CD69
Host
Ctrl
→ F5 Rag1-/-
B6 DP1
→ B6
E
Fold increase over host DP1
Host
→ F5 Rag1-/-
→ B6
10
5
0
1
2
3
4
5
Host CD8SP
Day of transfer

Figure 4.10 A model for the signalling characteristics directing phenotypic progression of positively selecting thymocytes

Based on the data in chapter 3-4, we have hypothesised the following signalling requirements directing the phenotypic progression of MHC-I (blue) or MHC-II (orange) restricted positively selecting thymocytes. All cells initially reside in the $TCR^{lo}CD5^{lo}$ DP1 population (non-selecting, black). Upon initiation of positive selection through MHC recognition, cells phenotypically transit to the $TCR^{int}CD5^{hi}$ DP2 population. DP2 thymocytes with a TCR restricted to MHC-II receive a relatively strong and consistent TCR signal due to properties conferred by the CD4 coreceptor. This permits lineage commitment, despite the fact that DP2 thymocytes are relatively insensitive to receiving TCR signals. In contrast DP2 thymocytes with a TCR restricted to MHC-I receive a relatively weak or intermittent TCR signal at the DP2 stage. This does not permit lineage commitment to occur, as DP2 cells are insensitive to receiving TCR signals. Instead cells phenotypically progress to the DP3 stage where they increase TCR and Zap70 expression and reduce CD5 expression. This now permits strong TCR signalling, despite the weak avidity/intermittent signalling properties conferred by the CD8 coreceptor. A strong TCR signal in the DP3 population instructs CD8 lineage commitment and thus by regulating TCR signalling sensitivity, strong/consistent and weak/intermittent signals can be discerned at different timepoints. Furthermore, positive autoregulation of Zap70 by the TCR may further differentiate strong and weak TCR signals.


CD5
DP2
MHC-II
Strong/Consistent TCR
Signal
CD4SP
Day 2
Lineage
Commitment
DP1
MHC-I
Weak/
Intermittent
TCR
Signal
DP3
Strong
Signalling
CD8SP
Day 4
TCR
Zap70
DP gated
SP gated

Chapter 5

The Impact of the Positive Selection Signal on Naïve T-cell Homeostasis

5.1 Introduction

Lymphocyte numbers are maintained at a constant level, despite continuous inputs of new cells and outputs via death or differentiation. The maintenance of cell numbers is achieved through the regulation of homeostatic survival factors, which become rate limiting if cell numbers are high or drive generation of new cells if in excess. Thus lymphocytes have the ability to sense whether the surrounding environment is lymphopenic or lymphoreplete. Essential lymphocyte survival factors include signals through the antigen receptor and signals mediated by cytokines (reviewed in (Freitas & Rocha, 2000)).

In the case of T-cells, homeostatic antigen receptor signals are transduced by the TCR (Introduced in **Chapter 3**, section **3.1**). Furthermore gamma chain (γc) cytokine signals, and in particular those mediated by IL7 are also essential for T-cell homeostasis (reviewed in (Jameson, 2002)). The IL7 receptor alpha chain (IL7rα) is constitutively associated with Janus kinase (JAK)1, whereas the γc is associated with JAK3 (reviewed in (Jiang *et al.*, 2005)). IL7 signals are transduced following crosslinking of the IL7rα and γc subreceptors, thus

bringing JAK1 and JAK3 into close proximity with one another, and leading to their mutual phosphorylation and activation (Foxwell *et al.*, 1995). Activated JAKs subsequently phosphorylate IL7rα, providing docking sites to recruit STAT5, which itself is a target of JAK kinase activity. Phosphorylated STAT (pSTAT)5 subsequently translocates to the nucleus, where it induces gene transcription (reviewed in (Jiang *et al.*, 2005)). A key pSTAT5 target is the antiapoptotic gene B-cell leukaemia/lymphoma 2 (Bcl2), which in part confers IL7 induced survival signal (Jiang *et al.*, 2004; Kim *et al.*, 1998b; von Freeden-Jeffry *et al.*, 1997).

Whilst ablation of MHC-II and MHC-I leads to a profound block in CD4 and CD8 lineage development respectively (Koller *et al.*, 1990; Madsen *et al.*, 1999), IL7 or IL7rα-deficiency also leads to a severe reduction in thymopoiesis of both lineages (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995). Thus TCR and IL7 signals control the generation of new peripheral T-cells. Furthermore, both TCR and cytokine signals are necessary to maintain mature T-cell numbers. Ablation of peripheral TCR signalling through cre-mediated TCR deletion (Polic *et al.*, 2001), or through cessation of Lck expression in tetracycline inducible Lck mice leads to a progressive loss of CD4 and CD8 naïve T-cells (Seddon *et al.*, 2000). Similarly T-cells do not survive for prolonged periods in IL7-deficient hosts (Schluns *et al.*, 2000) and IL7 improves T-cell survival *in vitro* (Tan *et al.*, 2001). In addition, IL7 transgenic mice show increased naïve T-cell numbers, suggesting that IL7 levels limit T-cell population size in lymphoreplete hosts

(Mertsching *et al.*, 1995). Hence TCR and IL7 signalling is essential not only for the generation of T-cells, but also for persistence of naïve T-cells.

Similarly to that of T-cells, B-cell homeostasis depends on signals mediated by the B-cell antigen receptor (BCR). Mice deficient in IgM heavy chain expression, a component of the BCR on all developing B-cells, exhibit developmentally arrested B-cell development (Kitamura *et al.*, 1991). Furthermore, mx-cre-induced deletion of the surface immunoglobulin (Ig) on mature B-cells results in a progressive loss of these cells (Lam *et al.*, 1997). In addition to BCR signalling, B-cells depend on soluble pro-survival signals mediated by the tumour necrosis factor (TNF)-family member, B-cell activating factor (BAFF), that signals through the BAFF-receptor (BAFFR). Mice deficient in BAFF or BAFFR exhibit in a block in B-cell development (Schiemann *et al.*, 2001; Thompson *et al.*, 2001). Moreover, recent advances in the understanding of B-cell homeostasis suggest there is extensive crosstalk between the BCR and BAFFR signalling pathways (reviewed in (Cancro, 2009)). BCR signals were found to direct BAFFR upregulation during B-cell development (Smith & Cancro, 2003; Walmsley *et al.*, 2003). In addition, BCR signalling induces the upregulation of the NFκB protein p100, which is a rate limiting substrate for BAFFR signal transduction (Stadanlick *et al.*, 2008). Thus BCR signals augment BAFFR signalling. It has been suggested that when BAFFR is limiting in a lymphoreplete environment, this will ensure that BCR-signalled clones are more likely to respond to limiting BAFF signals and thus preferentially survive. Hence there is crosstalk between generalised and clonotypic homeostatic

signals in B-cells (reviewed in (Cancro & Kearney, 2004)). In contrast, the relationship between the TCR and Interleukin-7 receptor (IL7R) signals remains controversial.

Recent findings suggest that modulating IL7rα expression could be a key control point for integrating TCR and IL7 signals. Excess IL7 signalling causes a downregulation of IL7rα, whereas IL7rα expression is upregulated when IL7 is limiting (Park *et al.*, 2004). Park et al. suggest that TCR signals block IL7 signalling, leading to an upregulation of the IL7rα that subsequently permits increased IL7 signalling (Park *et al.*, 2007). However, it has been suggested more recently that TCR signals actually augment IL7R function, by recruiting IL7rα and γc subreceptors to lipid rafts (Cho *et al.*, 2010). Furthermore, in the absence of peripheral TCR signalling IL7rα expression was decreased, suggesting a complex homeostatic relationship exists between the TCR and IL7rα signalling pathways (Takada & Jameson, 2009). Importantly, the molecular and genetic control mechanisms underlying these processes remain to be identified.

During T-cell development IL7rα is expressed on DN thymocytes, before expression is extinguished at the DP stage. IL7rα is subsequently re-expressed on positively selecting thymocytes, thus suggesting that the TCR-mediated positive selection signals can control IL7rα expression in developmentally immature T-cells (Akashi *et al.*, 1998; Sudo *et al.*, 1993). Whilst factors controlling the re-expression of IL7rα remain obscure, studies in conditional

knockout mice have identified some potential candidates. For example CD4-cre-mediated deletion of forkhead box O1 (Foxo1) resulted in defective IL7rα expression in thymic and peripheral SP populations (Kerdiles *et al.*, 2009). In addition GA-binding protein (Gabp) controlled IL7rα expression in DN cells and the thymic lymphoma derived EL4 cell line (Xue *et al.*, 2004). Interestingly, DP cells deficient for both Runx1 and Runx3 failed to upregulate IL7rα following positive selection (Egawa *et al.*, 2007). Runx factors are also essential for CD4 silencing and CD8 lineage commitment, suggesting they are functionally regulated during positive selection (Sato *et al.*, 2005; Taniuchi *et al.*, 2002; Woolf *et al.*, 2003). However, whether positive selection TCR signalling directly influences these factors, and which of these factors are necessary and/or sufficient for IL7rα re-expression remains to be determined. In addition, it is unknown if IL7rα is regulated in the same way during thymic development as it is in naïve peripheral T-cells.

Whilst there is evidence that IL7rα expression and/or function is modulated by TCR signalling in the periphery, the molecular mechanisms of this process remain to be elucidated. Furthermore, it is unknown whether developmental TCR signals can directly influence IL7rα re-expression following positive selection. Given that IL7rα is downregulated at the DP stage, and re-expressed following positive selection, we sought to investigate the relationship between positive selection TCR signalling and IL7rα re-expression.

5.2 Results

5.2.1 Disparate re-expression of IL7rα in CD4 and CD8 SP thymocytes

In **Chapter 1** we identified that CD4 and CD8 lineages develop with temporally distinct kinetics. Furthermore there is evidence that CD4 development is instructed by strong/consistent signalling whereas CD8 development is associated with weak/intermittent signals (reviewed in (Hogquist, 2001; Singer *et al.*, 2008)). Therefore, we asked whether IL7rα expression differed in CD4 and CD8 lineage cells. We examined IL7rα expression on B6 thymocytes and peripheral LN T-cells (LNTs) by flow cytometry. B6 $CD25^{-}CD44^{lo}TCR^{+}CD5^{+}$ (naïve) CD4 and CD8 LNTs were found to express similar levels of IL7rα (**Figure 5.1 A**). In the thymus, IL7rα was undetectable on non-selecting DP1 thymocytes. In contrast, IL7rα was expressed on SP thymic populations, with CD4 SP cells expressing relatively higher levels of IL7rα compared to CD8 SP cells (**Figure 5.1 B**, top). This suggested that IL7rα re-expression levels in the thymus correlated with the positive selection TCR signalling strength in B6 mice.

5.2.2 Suboptimal positive selection signalling correlates with a defect in IL7rα expression

Based on IL7rα re-expression patterns in B6 thymocytes, we hypothesised that the positive selection signal strength instructed IL7rα re-expression levels. To investigate this possibility, we examined IL7rα expression levels in mice that exhibit defects in TCR signalling. We first examined IL7rα expression in SKG

mice, which have a W163C mutation in the carboxy-terminal SH2 domain of Zap70, resulting in a reduction of Zap70 function (Sakaguchi *et al.*, 2003). These mice exhibit an equal defect in CD4 and CD8 SP lineage development, suggesting that reduced TCR signalling affected both lineages equally (**Chapter 3**, **Figure 4.5**). SKG mice showed a reduction in IL7rα re-expression in both CD4 and CD8 SP thymocyte and naïve LNT compartments, compared to BALB/c controls (**Figure 5.1 C**). Typically IL7rα expression was reduced to approximately 60-70% of BALB/c expression, representing a significant reduction in both CD4 and CD8 LNT populations (**Figure 5.1 D**) (CD4: $p \leq 0.001$, CD8: $p \leq 0.001$, Student's t-test BALB/c n=12, SKG n=12). We found no significant difference between the CD8:CD4 IL7rα MFI ratio, in SKG compared to BALB/c controls (p=ns, Student's t-test BALB/c n=12, SKG n=12) (**Figure 5.1 E**), suggesting that the defect in IL7rα expression was equal in LNTs of both lineages.

We secondly examined IL7rα expression in TetZap70^{on} mice, which have a specific defect in TCR signalling in the CD8 lineage relative to a more minor defect in the CD4 lineage, resulting from dysregulated developmental expression of Zap70 (**Chapter 4**) (Saini *et al.*, 2010). In these mice, both thymic and peripheral CD4 and CD8 cells exhibited reduced IL7rα expression compared to B6 controls (**Figure 5.1 F**). Typically IL7rα expression was reduced to approximately 70% of B6 levels in the CD4 lineage and 50% of B6 levels in the CD8 LNTs (CD4: $p \leq 0.001$, CD8: $p \leq 0.001$, Student's t-test B6 n=7, TetZap70^{on} n=7) (**Figure 5.1 G**). Interestingly, we found that the CD8:CD4

IL7rα MFI ratio was significantly reduced in the LNT populations of TetZap70 mice ($p \leq 0.001$, Student's t-test B6 n=7, TetZap70^{on} n=7) (**Figure 5.1 H**). Thus peripheral CD8 SP cells from TetZap70^{on} mice exhibited a greater defect in IL7rα expression relative to CD4 SPs. Our results suggest that defective positive selection TCR signalling proportionally correlates with a corresponding defect in IL7rα re-expression.

5.2.3 Low avidity TCR transgenic T-cells have a reduced expression of IL7rα

SKG and TetZap70^{on} mice both exhibit defects in TCR signalling at the level of Zap70 function/expression. We next investigated whether T-cell clones exhibiting a low avidity for spMHC also had reduced IL7rα expression. To do so, we examined IL7rα expression in thymocytes and naïve LNTs from TCR transgenic mice that have different avidities for spMHC. F5 and OT-I T-cells express MHC-I restricted TCRs, and therefore on a *Rag1*$^{-/-}$ background only generate CD8 SP lineage cells (**Figure 5.2 A**) (Hogquist *et al.*, 1994; Mamalaki *et al.*, 1993). We compared levels of CD5 on F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ T-cells, since CD5 expression is known to correlate with the TCR avidity to spMHC in the periphery (Azzam *et al.*, 2001; Smith *et al.*, 2001). Whereas B6 naïve CD8 SP cells showed a relatively broad range of CD5 expression, F5 *Rag1*$^{-/-}$ cells expressed relatively low levels of CD5. In contrast OT-I *Rag1*$^{-/-}$ cells exhibited relatively high expression of CD5 (**Figure 5.2 B**). This suggested that in the periphery, F5 *Rag1*$^{-/-}$ T-cells received weak signals from spMHC compared to OT-I *Rag1*$^{-/-}$ T-cells.

We next asked whether the differences in TCR:spMHC avidity were also reflected during positive selection of F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ thymocytes. To address this, thymic phenotypes of F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice were examined by flow cytometry. The CD4 versus CD8 profile of F5 *Rag1*$^{-/-}$ mice was distinct from that of OT-I *Rag1*$^{-/-}$ mice, largely due to the absence of a CD4^{hi}CD8^{lo} population of positively selecting thymocytes (**Figure 5.2 C**). It has been suggested that the CD4^{hi}CD8^{lo} population represents a developmental intermediate for high avidity MHC-I-restricted thymocytes (reviewed in (Hogquist, 2001)), implying that the OT-I *Rag1*$^{-/-}$ thymocytes receive a stronger positive selection signal relative to F5 *Rag1*$^{-/-}$ thymocytes. CD5 and CD69 are upregulated on positively selecting thymocytes, and correlate with positive selection signalling strength (Azzam *et al.*, 1998; Swat *et al.*, 1993). Thus we investigated the expression of these factors in B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. B6 DPs exhibited a broad range of CD5 expression, including a shoulder of higher CD5 expression that represents the positively selecting cells (**Figure 5.2 D**). Transgenic mice have prearranged TCRs and undergo positive selection homogenously, thus the majority of DP thymocytes in transgenic mice are CD5^{hi} (Itano & Robey, 2000; Wong *et al.*, 2000). However, OT-I *Rag1*$^{-/-}$ DPs were found to express higher levels of CD5 relative to F5 *Rag1*$^{-/-}$ DPs (**Figure 5.2 D**). CD69 was absent from B6 DP1 cells, however it was induced on TCR^{hi}CD5^{hi} positively selecting DPs. Furthermore CD69 expression was highest on B6, intermediate on OT-I *Rag1*$^{-/-}$ and low on F5 *Rag1*$^{-/-}$ TCR^{hi}CD5^{hi} DP thymocytes (**Figure 5.2 E**). Taken together, these data suggest that OT-I *Rag1*$^{-/-}$ thymocytes receive a stronger positive selection signal than

F5 *Rag1*$^{-/-}$ thymocytes, which correlates with the avidity of these cells for spMHC in the periphery.

Our next question was whether the positive selection signal strength in the thymus correlated with differences in IL7rα re-expression levels/kinetics. To answer this, we investigated the expression of IL7rα in DP1-3 and in thymic and peripheral CD8 SP populations of B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice by flow cytometry. IL7rα was expressed at background levels in the DP1 and DP2 populations of B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice (**Figure 5.2 F**, top row). We found that IL7rα was first re-expressed in the DP3 populations of B6 and OT-I *Rag1*$^{-/-}$ mice, with similar abundance. In contrast IL7rα expression remained at background levels in F5 *Rag1*$^{-/-}$ DP3 thymocytes (**Figure 5.2 F**, second row). IL7rα expression was lower on OT-I *Rag1*$^{-/-}$ CD8 SPs relative to the equivalent B6 population. Surprisingly, IL7rα expression was undetectable in CD8 SP cells from F5 *Rag1*$^{-/-}$ mice (**Figure 5.2 F**, third row). Thus the positive selection signal strength correlated well with IL7rα in terms of both thymic re-expression kinetics and abundance. We next examined expression of IL7rα in peripheral CD8 SPs from B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. Interestingly, despite lacking IL7rα expression in the thymus, F5 *Rag1*$^{-/-}$ mice did re-express IL-7rα, albeit at low levels, in CD8 LNTs relative to levels found in B6 CD8 LNTs. In contrast, OT-I *Rag1*$^{-/-}$ CD8 LNTs expressed higher levels of IL7rα than F5 *Rag1*$^{-/-}$ CD8 LNTs, although this still represented a slight reduction compared to levels on WT CD8 T-cells (**Figure 5.2 F**, bottom row). The differences between F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ CD8 LNT IL7rα abundance

was cell intrinsic, as differences persist when F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ LNTs were competed with B6 T-cells in mixed BM chimeras (Sinclair et al., manuscript submitted). Thus avidity for spMHC correlated with peripheral IL7rα expression in TCR transgenic mice.

We next asked whether differences in IL7rα surface re-expression levels and kinetics correlated with Il7r mRNA abundance. DP2, DP3 and CD8 SP thymic populations were purified by cell sorting to high (≥95%) purity from F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice, before mRNA was isolated. mRNA was additionally isolated from total LNTs of these mice. We found that Il7r mRNA expression mirrored protein expression. Whereas OT-I *Rag1*$^{-/-}$ cells expressed high levels of Il7r in DP3 thymocytes, Il7r was only expressed at low levels in F5 *Rag1*$^{-/-}$ cells. Il7r was however highly expressed in LNTs of both F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice (**Figure 5.2 G**). Taken together, these data suggests that suboptimal positive selection signalling results in a failure to upregulate *Il7r* transcription in DP3 and CD8 SP cells from F5 *Rag1*$^{-/-}$ mice.

5.2.4 Runx expression precedes IL7rα expression in F5 *Rag1*$^{-/-}$ thymocytes

Several transcription factors have been implicated in regulating *Il7r* transcription in a variety of T-cell developmental stages. We next investigated whether such factors were differently regulated in DP2, DP3, CD8 SP and LNT populations from F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. Mice with a conditional deletion of *Foxo1* at the DP stage showed reduced IL7rα expression levels in both CD4

and CD8 SP thymocytes and naïve LNTs (Kerdiles *et al.*, 2009). Gabp consists of alpha and beta subunits, and was shown to regulate IL7rα expression in a thymic lymphoma derived cell line (EL4) and primary DN thymocytes (Xue *et al.*, 2004). We found that Foxo1 mRNA was progressively upregulated between DP2-CD8 LNT populations in both F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice (**Figure 5.3 A**). GA-binding protein β-subunit 1 (*Gabpb1*) that encodes the Gabp β-subunit exhibited a contrasting pattern of expression, as it was progressively downregulated between DP2-LNT populations. However, we again saw no difference in expression between F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ populations (**Figure 5.3 B**).

Mice with conditional deletions of Runx1 and Runx3 proteins at the DP stage show defects in Il7r re-expression in CD4 and CD8 SP lineages respectively (Egawa *et al.*, 2007). We investigated whether Runx factors may be differently regulated between F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. Runx1 was expressed at slightly elevated levels in the DP2 population of F5 *Rag1*$^{-/-}$ relative to OT-I *Rag1*$^{-/-}$ mice. In F5 *Rag1*$^{-/-}$ mice, Runx1 was reduced to background levels in the DP3 and CD8 SP populations. In OT-I *Rag1*$^{-/-}$ mice Runx1 expression was elevated by approximately 2-3 fold between the DP2 and DP3 populations, before falling to background levels in the thymic and peripheral CD8 SP populations (**Figure 5.3 C**). Runx3 was expressed at similar levels in the DP2 population of F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. In F5 *Rag1*$^{-/-}$ mice, whilst Runx3 levels remained low in the DP3 population, Runx3 showed an approximately 2-fold increase between DP3 and CD8 SP populations. The CD8 SP LNT

population from F5 *Rag1*$^{-/-}$ mice expressed similar levels of Runx3 as the thymic CD8 SP compartment. In contrast Runx3 expression was increased by approximately 8-fold between DP2 and DP3 populations of OT-I *Rag1*$^{-/-}$ mice. Runx3 expression remained at this elevated level in the thymic CD8 SP population and was lower in CD8 SP LNTs, where expression was similar to that seen in F5 *Rag1*$^{-/-}$ LNTs (**Figure 5.3 D**). Taken together, these data indicated that lower expression of Runx1 and Runx3 in the DP3 and CD8 SP thymic compartments correlated with reduced IL7rα expression in thymic and peripheral T-cell populations in F5 *Rag1*$^{-/-}$ relative to OT-I *Rag1*$^{-/-}$ mice.

5.2.5 IL7 not TCR signalling predominantly modulates peripheral IL7rα expression and function

Our data thus far correlated reduced TCR signalling during positive selection with a reduction in IL7rα re-expression. In peripheral T cells however, IL7rα is upregulated in the absence of IL7-mediated signalling and downregulated following IL7 signalling (Park *et al.*, 2004). TCR signalling has been suggested to increase IL7R expression by blocking IL7R signal transduction through an indirect mechanism (i.e. reducing IL7-mediated signalling) (Park *et al.*, 2007). We therefore sought to test whether IL7 or TCR signalling was the predominant factor determining IL7rα expression in naïve LNTs. To do this we transferred MHC-I-restricted TCR transgenic lymphocytes to different host environments that allowed increased or decreased IL7R and TCR signalling. We hypothesised that T-cell-deficient *Rag1*$^{-/-}$ *b2m*$^{-/-}$ hosts would have high levels of excess IL7, as they lack peripheral T-cells. However these hosts would not

permit TCR signalling for CD8 LNTs as they lack the β_2m component of MHC-I. In contrast, transfer to *Rag1*$^{-/-}$ hosts would permit high levels of both IL7 and TCR signalling. TCR transgenic mice are partially lymphopenic and thus have intermediate levels of IL7 relative to Rag-deficient and WT mice (Ge *et al.*, 2004). However, high levels of intraclonal competition exist in such an environment, suggesting TCR signals may be limited. Finally we hypothesised that following transfer of MHC-I-restricted TCR transgenic thymocytes to B6 hosts, IL7 signals would be rate-limiting, whilst a reduction in intraclonal competition would permit an increase in TCR signalling. The hypothesised relative levels of IL7R and TCR signals are summarised in **Table 5.1**.

Table 5.1 Hypothesised TCR and IL7 signals received by donor MHC-I-restricted TCR transgenic LNTs in different host environments

Host	TCR Signalling	IL7 Signalling
Rag1$^{-/-}$ *b2m*$^{-/-}$	-	+++
Rag1/2$^{-/-}$	+++	+++
TCR transgenic	+	++
B6	++	+

We first investigated the effects of transferring weak avidity F5 *Rag1*$^{-/-}$ lymphocytes to different host environments. Total LNTs from F5 *Rag1*$^{-/-}$ mice were isolated and $5x10^6$ cells were transferred intravenously (I.V.) to *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag1*$^{-/-}$, F5 *Rag1*$^{-/-}$ or B6 congenically marked hosts for three

days. F5 *Rag1*$^{-/-}$ donor cells were re-isolated and found to be of a naïve CD25^{lo}CD44^{lo} phenotype, irrespective of the host strain (**Figure 5.4 A**). We next examined the level of CD5 on donor LNTs to determine the level of TCR stimulation by spMHC in each host. Cells transferred to the *Rag1*$^{-/-}$ *b2m*$^{-/-}$ hosts exhibited low levels of CD5, suggesting that TCR signalling was reduced or absent. In contrast, F5 *Rag1*$^{-/-}$ cells transferred to both *Rag1*$^{-/-}$ and B6 hosts showed a relative increase in CD5 expression suggesting that TCR signalling was increased in these donors relative to cells in F5 *Rag1*$^{-/-}$ controls (**Figure 5.4 B**). These results fit with the predicted levels of TCR signalling in different host environments (**Table 5.1**).

We next determined IL7rα expression on donor lymphocytes to see whether there was any evidence for TCR signalling regulating peripheral IL7rα abundance. F5 *Rag1*$^{-/-}$ donor LNTs transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$ and *Rag1*$^{-/-}$ hosts expressed similar or slightly reduced levels of IL7rα compared to donor LNTs transferred to F5 *Rag1*$^{-/-}$ control hosts. In contrast, those donor cells transferred to B6 hosts upregulated IL7rα expression, although it was not expressed as highly as in endogenous B6 CD8 SP host LNTs (**Figure 5.4 C**). Therefore IL7rα expression correlated better with IL7 availability rather than spMHC availability *in vivo*.

Recently, Cho et al. reported that TCR signalling augmented IL7 signalling, through recruitment of the IL7rα and the γc to lipid rafts (Cho *et al.*, 2010). Consequently, we tested the downstream function of IL7rα on F5 *Rag1*$^{-/-}$ donor

LNTs transferred to different hosts, by stimulating cells with IL7 for 30 minutes and measuring the induction of pSTAT5. pSTAT5 induction was found to closely correlate with IL7rα expression levels. F5 *Rag1*$^{-/-}$ donor cells transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag1*$^{-/-}$ and F5 *Rag1*$^{-/-}$ hosts showed relatively low levels of pSTAT5 induction, relative to B6 hosts (**Figure 5.4 D**). IL7rα function therefore correlated with IL7rα expression levels and not previous TCR signalling.

The F5 *Rag1*$^{-/-}$ mice have a weak avidity for spMHC and may not be representative of higher avidity clones. We therefore wanted to confirm that peripheral IL7rα expression correlated with IL7 availability and not spMHC availability in OT-I *Rag1*$^{-/-}$ mice, which have a high avidity for spMHC (**Figure 5.2 A-E**) (Ge *et al.*, 2004). We transferred 5x10^6 OT-I *Rag1*$^{-/-}$ donor LNTs to congenically marked *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag2*$^{-/-}$ or B6 hosts, that together permitted different levels of IL7/TCR signalling. In addition, we examined pre-transferred OT-I *Rag1*$^{-/-}$ LNTs as controls (**Table 5.1**). Similar to the F5 *Rag1*$^{-/-}$ donor LNTs, OT-I *Rag1*$^{-/-}$ LNTs were of a naïve phenotype following re-isolation three days after transfer, irrespective of the host environment (**Figure 5.5 A**). Relative to pre-transferred OT-I *Rag1*$^{-/-}$ cells, OT-I *Rag1*$^{-/-}$ donors transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$ hosts showed reduced expression of CD5. In contrast, those donor cells transferred to *Rag2*$^{-/-}$ or B6 hosts showed elevated levels of CD5 (**Figure 5.5 B**). These findings confirmed our results following transfer of F5 *Rag1*$^{-/-}$ LNTs to different hosts, in that IL7rα expression inversely correlated with IL7 availability *in vivo*.

We next investigated whether IL7rα expression was differently expressed on donor OT-I *Rag1*$^{-/-}$ LNTs three days after transfer. We found that relative to the intact OT-I *Rag1*$^{-/-}$ mice, the OT-I *Rag1*$^{-/-}$ donor LNTs transferred to lymphopenic *Rag1*$^{-/-}$ *b2m*$^{-/-}$ or *Rag2*$^{-/-}$ hosts had relatively low levels of IL7rα expression. In contrast, those cells transferred to B6 mice had similar levels of IL7rα, relative to the intact OT-I *Rag1*$^{-/-}$ mice (**Figure 5.5 C**). Once more, IL7rα expression was found to correlate with IL7 signalling, as OT-I *Rag1*$^{-/-}$ LNTs transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$ and *Rag2*$^{-/-}$ hosts showed lower pSTAT5 induction following 30 minutes of IL7 treatment, when compared to intact OT-I *Rag1*$^{-/-}$ mice, or OT-I *Rag1*$^{-/-}$ donor cells transferred to B6 hosts. Thus results with strong avidity OT-I *Rag1*$^{-/-}$ donor lymphocytes parallel results seen following transfer of weak avidity F5 *Rag1*$^{-/-}$ lymphocytes. These data suggest that peripheral IL7 signals predominantly modulate IL7rα expression levels in the periphery, irrespective of TCR signalling strength.

5.2.6 Increasing the positive selection signalling strength restores IL7rα expression in F5 *Rag1*$^{-/-}$ thymocytes

Our results suggested that different mechanisms exist in the thymus and the periphery to control IL7rα expression levels. In the periphery, we found that predominantly IL7 signals and not TCR signals control IL7rα expression on peripheral naïve LNTs (**Figure 5.1**, **Figure 5.2**). This contrasted with the hypothesis that TCR signals influence peripheral IL7rα expression levels (Park *et al.*, 2007). In contrast, the weakly selecting F5 *Rag1*$^{-/-}$ thymocytes exhibited no detectable IL7rα re-expression, suggesting that IL7 signals were unlikely to

be influencing this process (**Figure 5.2 F**). Instead, these findings were suggestive that lower IL7rα expression in F5 *Rag1*$^{-/-}$ mice was a direct result of reduced TCR signalling during thymic development. Thus we asked whether increasing the positive selection signal in F5 *Rag1*$^{-/-}$ thymocytes could rescue IL7rα expression in F5 *Rag1*$^{-/-}$ cells. In the periphery, reduction of intraclonal competition permitted increased TCR signalling as measured by CD5 expression, when F5 *Rag1*$^{-/-}$ cells were transferred to B6 hosts (**Figure 5.4 B**). We hypothesised that reducing the clonal competition of F5 *Rag1*$^{-/-}$ thymocytes during positive selection may increase the positive selection signal strength and permit us to investigate the effects on IL7rα expression. F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} thymocytes are arrested at the DP1 pre-positively selecting stage (**Chapter 3**, **Figure 3.8 D**). We therefore used these mice as a source of clonotypic TCR transgenic DP1 arrested thymocytes. We intrathymically transferred 1-2x10^{6} F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} donor thymocytes to β_2m-sufficient B6 CD45.1^{+} hosts that allowed an environment of low clonal competition, or to F5 *Rag1*$^{-/-}$ CD45.1^{+} controls. Following intrathymic injection transferred donor thymocytes were re-isolated after 2-5 days and examined by flow cytometry. Analysis of CD4 and CD8 expression revealed modulation of coreceptor expression levels on days two and three, which was more apparent in those cells transferred to B6 hosts compared to F5 *Rag1*$^{-/-}$ hosts. However, on days four and five both B6 and F5 *Rag1*$^{-/-}$ host environments permitted the development of CD8 SPs (**Figure 5.6 A**). Examination of TCR and CD5 expression on transferred thymocytes revealed cells underwent a progressive phenotypic progression from DP2 > DP3 > CD8 SP, irrespective of the host

environment, and with similar kinetics as described for B6 thymocytes (**Chapter 3**, **Figure 3.8**) (**Figure 5.6 B**).

We next asked whether transfer of F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes to B6 or F5 *Rag1*$^{-/-}$ hosts impacted on the positive selection signal strength. Expression of the activation marker CD69 on donor thymocytes was therefore determined following intrathymic transfer. Between days two and five, CD69 expression was consistently higher on those F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes transferred to B6 hosts, as opposed to F5 *Rag1*$^{-/-}$ hosts. This finding reflected differences in the host TCR^{hi}CD5^{hi} DP populations, as B6 host thymocytes had higher levels of CD69 relative to F5 *Rag1*$^{-/-}$ hosts, further suggesting that these differences were intrinsic to the host environment (**Figure 5.6 C**). Thus, transfer of F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes to B6 hosts resulted in quantitatively elevated positive selection signalling compared with transfer to F5 *Rag1*$^{-/-}$ hosts. These differences reflected the comparatively lower levels of intraclonal competition in B6 relative to F5 *Rag1*$^{-/-}$ host environments.

Finally we wished to determine whether the augmentation of the positive selection signal in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes could rescue IL7rα re-expression levels or kinetics. IL7rα was absent on F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes two days following transfer, irrespective of recipient mouse. Interestingly, IL7rα was expressed on donor thymocytes transferred to B6 hosts on day three. We subsequently saw a progressive upregulation of IL7rα, so that by day five, donor F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes expressed similar levels of IL7rα compared to the B6 host CD8 SP population. In sharp contrast, we saw

no expression of IL7rα on donor thymocytes in F5 *Rag1*$^{-/-}$ hosts by day five, reflecting the absence of IL7rα expression on host F5 *Rag1*$^{-/-}$ thymocytes (**Figure 5.6 D**). These data indicated that augmenting the positive selection signal strength positively influences IL7rα re-expression levels and kinetics. Such a mechanism may influence the homeostatic survival potential of T-cells, as differences in IL7rα expression are likely to affect the ability of cells to compete with one another for peripheral IL7 survival signals.

5.3 Discussion

We have described a novel homeostatic mechanism, whereby the strength of the positive selection signal dictates IL7rα expression levels. We first found that IL7rα expression was sub-optimally re-expressed in mice exhibiting defects in TCR signalling machinery, or in transgenic T-cells receiving a weak positive selection signal. The defect in IL7rα re-expression correlated with a lower/delayed expression of Runx1 and Runx3 in the low avidity TCR transgenic F5 *Rag1*$^{-/-}$ mice compared to the OT-I *Rag1*$^{-/-}$ mice. In contrast, IL7 and not TCR signalling predominantly controlled IL7rα expression levels in the periphery. This suggested the control of IL7rα expression by the TCR was a distinct and unique property of the positive selection signal. Finally, we showed that reducing clonal competition between positively selecting F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes not only augmented the positive selection signal, but also restored IL7rα re-expression kinetics and abundance in positively selected thymocytes. We therefore suggest a mechanism whereby positive selection TCR signalling confers a greater survival potential to thymocytes with a strong avidity for spMHC, by instructing cells to express higher levels of IL7rα.

Positive selection of CD4 SP cells is associated with stronger TCR signalling relative to selection of CD8 SP cells (reviewed in (Hogquist, 2001)). Our finding that CD4 SP thymocytes also express higher levels of IL7rα in the thymus led us to investigate whether positive selection signal strength impacted on IL7rα re-expression (**Figure 5.1 B**). We found that SKG mice, which have a mutation

in Zap70 that reduces TCR signalling, had reduced IL7rα expression in both CD4 and CD8 LNTs (**Figure 5.1 C-E**). In contrast, TetZap70^{on} mice showing a specific defect in CD8 lineage TCR signalling had a correspondingly greater defect in peripheral CD8 SP IL7rα expression (**Figure 5.1 F-H**). Similarly the low avidity F5 *Rag1*$^{-/-}$ mice showed defective IL7rα re-expression relative to the high avidity OT-I *Rag1*$^{-/-}$ mice, in terms of mRNA and protein abundance (**Figure 5.2**). Importantly this defect in IL7rα could be corrected by augmenting the positive selection signal, by reducing the intrathymic clonal competition (**Figure 5.6**). Thus we conclude that relatively weak positive selection signal strength confers an IL7rα^{lo} phenotype to selected T-cells. It has been described that IL7rα is upregulated on positively selecting thymocytes (Akashi *et al.*, 1998; Sudo *et al.*, 1993) and is upregulated after treatment of DP thymocytes by concentrations of PMA and ionomycin that mimic the positive selection signal (Brugnera *et al.*, 2000). Our results extend these findings, by suggesting that IL7rα expression levels are linked to the positive selection signal strength of the TCR. Indeed such a finding is reminiscent of B-cell positive selection, whereby decreased BCR signalling strength correlates with reduced BAFFR expression (Walmsley *et al.*, 2003).

In contrast, we found that IL7 signals predominated over TCR signals in controlling peripheral IL7rα expression (**Figure 5.4-5.5**). This supports the argument that a predominating role of TCR signalling in controlling IL7rα expression is unique to the positive selection signal. IL7 signalling has been shown to downregulate of IL7rα in the periphery (Park *et al.*, 2004). However,

such a mechanism cannot account for all of our findings. For example, TetZap70^{on} thymocytes have a specific defect for IL7rα expression the CD8 lineage relative to the CD4 lineage (**Figure 5.1 F-H**), whilst the defect in F5 *Rag1*$^{-/-}$ T-cells is cell intrinsic, since it is present in mixed BM chimeras (Sinclair et al. Manuscript submitted).

A number of recent findings suggest that TCR and IL7 signal integration may be complex. Park et al. have also proposed the concept of coreceptor tuning in peripheral CD8 cells, whereby TCR signals block IL7 signalling, thus leading to an upregulation of IL7rα (Park *et al.*, 2007). Consequently IL7 signalling is increased, which feeds back to downregulate IL7rα and upregulated CD8α, thus increasing TCR signalling. In contrast TCR signalling did not affect CD4 expression (Park *et al.*, 2007). Paradoxically, Cho et al. have recently described that TCR signalling actually increased IL7 sensitivity, by inducing IL7rα and γc localisation in lipid rafts (Cho *et al.*, 2010). Furthermore, loss of TCR signalling mediated by transfer of naïve T-cells to MHC-I-deficient hosts resulted in decreased IL7rα expression within the CD8 lineage (Takada & Jameson, 2009). However, another conflicting finding showed loss of peripheral Lck signalling has no effect on IL7rα expression levels (Seddon *et al.*, 2003). Hence, the precise relationship between TCR signalling and IL7rα expression is unclear. In addition these studies cannot account for the complete absence of IL7rα expression in F5 *Rag1*$^{-/-}$ thymocytes (**Figure 5.2 F-G**). That these low avidity cells positively select to the CD8 lineage without re-expressing IL7rα suggests that IL7rα expression is indeed sensitive to TCR signalling quantity.

This characteristic appears to contradict the proposed role of IL7 signals in instructing the CD8 SP lineage decision (see below) (Brugnera *et al.*, 2000) (reviewed in (Singer *et al.*, 2008)). However, it remains to be investigated whether F5 *Rag1*$^{-/-}$ thymocytes are indeed completely insensitive to IL7 signalling, as their apparent lack of IL7rα implies.

Our data imply that transcriptional mechanisms underlie differences in IL7rα expression in F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice (**Figure 5.2 G**). A number of transcription factors have been implicated in controlling IL7rα expression in various cell types (reviewed in (Mazzucchelli & Durum, 2007)), some of which we examined. We found that Foxo1 was similarly expressed in both F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice (**Figure 5.3 A**). Kerdiles et al. found that CD4-Cre driven Foxo1 deficiency that deleted Foxo1 at the DP stage resulted in reduced IL7rα expression in thymic and peripheral SP cells (Kerdiles *et al.*, 2009). Our results suggest that in Foxo1-deficiency may affect maintenance rather than induction of IL7rα. Xue et al. showed that Gabp expression was essential for IL7rα expression in DN thymocytes and the EL4 cell line (Xue *et al.*, 2004). However, similar mRNA expression of Gabpb1 in F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ thymocytes suggest this factor may not be important for setting IL7rα re-expression levels. Alternatively, Gabp may be regulated in DP thymocytes by limiting the abundance of the Gabp α-subunit, the expression levels of which remain to be tested in F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ thymocytes.

Nevertheless, we did find that reduced IL7rα expression correlated with reduced Runx1 and Runx3 expression in F5 *Rag1*$^{-/-}$ cells (**Figure 5.3 C-D**). This fits with data from Egawa et al. whereby CD4-Cre-mediated deletion of Runx1 and Runx3 resulted in reduced IL7rα expression in CD4 and CD8 lineages respectively (Egawa *et al.*, 2007). A Runx1 binding site exists in the IL7rα promotor region, and mutation of this site in the transformed DP cell line, KKF, resulted in reduced IL7rα expression (Lee *et al.*, 2005). However it remains to be determined whether Runx1 and/or Runx3 physically interact with the IL7rα locus in primary positively selecting T-cells, and whether this is essential for IL7rα re-expression. The recent finding that Runx3 expression is upregulated *in vitro* following IL7 treatment further complicates the correlation between Runx1/3 and IL7rα expression (Park *et al.*, 2010). Our findings suggest that Runx3 upregulation can actually precede IL7rα induction, suggesting Runx3 also upregulated through IL7-independent mechanisms (**Figure 5.2 G**, **Figure 5.3 D**). It would be interesting to know whether Runx factors and IL7:IL7rα signalling can reciprocally regulate one another. Furthermore, Runx factors associate with the Brg/Brahma-associated factors (BAF) chromatin remodelling complex (reviewed in (Taniuchi & Littman, 2004)), thus comparisons of the chromatin configuration in F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ SP thymocytes will be a future goal for the lab. Finally, it is interesting that *Runx1*/*Runx3* gene deletion did not entirely ablate IL7rα re-expression (Egawa *et al.*, 2007). Gene expression analysis of F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ thymocytes may give insight into additional factors regulating IL7rα expression. In conclusion, we have identified a cellular mechanism whereby IL7rα

expression is set based on the positive selection signalling strength, however the genetic mechanism/s remain to be elucidated.

Following transfer to lymphoreplete B6 hosts, both F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ thymocytes upregulate IL7rα expression (**Figure 5.4 E**, **Figure 5.5 E**). This suggests that IL7 is not limiting in these transgenic mouse models. However, IL7 is likely to be limited in lymphoreplete WT mice, as IL7 transgenic mice have increased T-cell numbers (Mertsching *et al.*, 1995) and mice lacking either CD4 or CD8 lineages have similar numbers of T-cells compared to WT (Fung-Leung *et al.*, 1991; Rahemtulla *et al.*, 1991). Our findings lead us to propose a model whereby the positive selection signal strength controls IL7rα expression. Such a mechanism would serve to continually refine the peripheral T-cell pool, as weakly selected clones would be preferentially lost in conditions where IL7 was limiting. This could include low avidity clones, overrepresented clonotypes exhibiting high intraclonal competition for thymic TCR ligands, and those T-cells with defective TCR rearrangements or defects in TCR-associated signalling molecules. It remains to be tested whether IL7rα^{lo} cells will be efficiently purged from a lymphoreplete environment as compared with IL7rα^{hi} cells. Further studies will follow the fate of B6 IL7rα^{lo} and IL7rα^{hi} post selection thymocytes following I.V. transfer to congenic WT hosts. IL7 has been shown to enhance homeostatic proliferation (Tan *et al.*, 2001), so T-cell clones that have received a stronger positive selection signal may be more likely to undergo homeostatic proliferation during lymphopenia. Thus we suggest the IL7rα expression levels confer a memory of positive selection signal strength to a T-cell.

Our model suggests a mechanism of antigen receptor and cytokine signal integration that contrasts with that of B-cells. BCR signals control the expression of BAFFR (Smith & Cancro, 2003; Walmsley *et al.*, 2003), and in addition induce the upregulation of the NK-κB substrate p100, which is rate limiting in BAFFR signal transduction (Stadanlick *et al.*, 2008). We have identified that TCR signalling controls IL7rα expression levels during T-cell development. Although there is no evidence that TCR and IL7 signalling pathways show signal integration, it would be interesting to investigate whether such integration may occur post-transcriptionally, perhaps through the coregulation of early genes induced by TCR and IL7 signalling pathways. There is functional evidence for signal integration, as both IL7 and TCR signals can synergise to promote homeostatic proliferation (reviewed in (Surh & Sprent, 2008)).

In conclusion, the results presented in this chapter give further insight to T-cell homeostatic control mechanisms. Such findings may have implications for human diseases in which T-cell homeostasis is perturbed. Administration of exogenous IL7 has been trialled in acquired immunodeficiency syndrome (AIDS) patients (Levy *et al.*, 2009), whereas IL7 administration has been proposed as a treatment to boost T-cell numbers in elderly patients (reviewed in (Aspinall *et al.*, 2007)). Our findings suggest that such exogenous IL7 treatment may permit the continued survival of weaker avidity clones. It will be interesting to determine whether this is indeed the case and if so, whether this impacts on the functionality of the reconstituted T-cell pool.

Figure 5.1 Peripheral IL7rα expression correlates with the positive selection signalling strength

B6, BALB/c, SKG and TetZap70^{on} T-cells were analysed for the expression of CD4, CD8, TCR, CD5, CD25, CD44 and IL7rα by flow cytometry. (**A**) B6 LNs were analysed. Density plot shows CD4 against CD8 expression on naïve LN T-cells (CD25^{lo}CD44^{lo}TCR^{hi}CD5^{hi}). Histogram shows IL7rα expression on naive CD4 SP (red line) and CD8 SP (blue line) (CD4 and CD8 SP gating is shown in left plot). (**B**) B6 thymi were analysed. Density plot shows CD4 against CD8 expression on live thymocytes. Histogram shows IL7rα expression on DP1 (grey fill), CD4 SP (red line) and CD8 SP (blue line). Data is representative of ≥3 independent experiments. (**C**) Histograms show the expression IL7rα on naïve CD4 SP T-cells from BALB/c (black line) or SKG (red line) thymi and LNs (top row), and naïve CD8 SP T-cells derived from BALB/c (black line) or SKG (blue line) thymi and LNs (bottom row). BALB/c DP1 cells (grey fill) are shown as a negative control. (**D**) The bar chart shows the mean IL7rα MFI on naïve CD4 SP (red) and CD8 SP (blue) LNTs from a cohort of SKG mice, as a frequency of the IL7rα MFI on equivalent populations from BALB/c mice. *** p≤0.001 (Student's t-test, BALB/c n=12, SKG n=12). (**E**) The bar chart shows the CD8:CD4 ratio of IL7rα MFI on BALB/c and SKG LNTs. p=ns (Student's t-test, BALB/c n=12, SKG n=12). (**F**) Histograms show the expression IL7rα on naïve CD4 SP T-cells derived from B6 (black line) or TetZap70^{on} (red line) thymi and LNs (top row), and naïve CD8 SP T-cells derived from B6 (black line) or TetZap70^{on} (blue line) thymi and LNs (bottom row). B6 DP1 cells (grey fill) are shown as a negative control. (**G**) The bar

chart shows the mean IL7rα MFI on naïve CD4 SP (red) and CD8 SP (blue) LNTs from a cohort of TetZap70^{on} mice, as a frequency of the IL7rα MFI on equivalent populations from B6 mice. *** p≤0.001 (Student's t-test, B6 n=7, TetZap70^{on} n=7). (**H**) The bar chart shows the CD8:CD4 ratio of IL7rα MFI on B6 and TetZap70^{on} LNTs. *** p≤0.001 (Student's t-test, B6 n=7, TetZap70^{on} n=7). Error bars represent sd. The data in (**F-H**) were obtained my Moni Saini

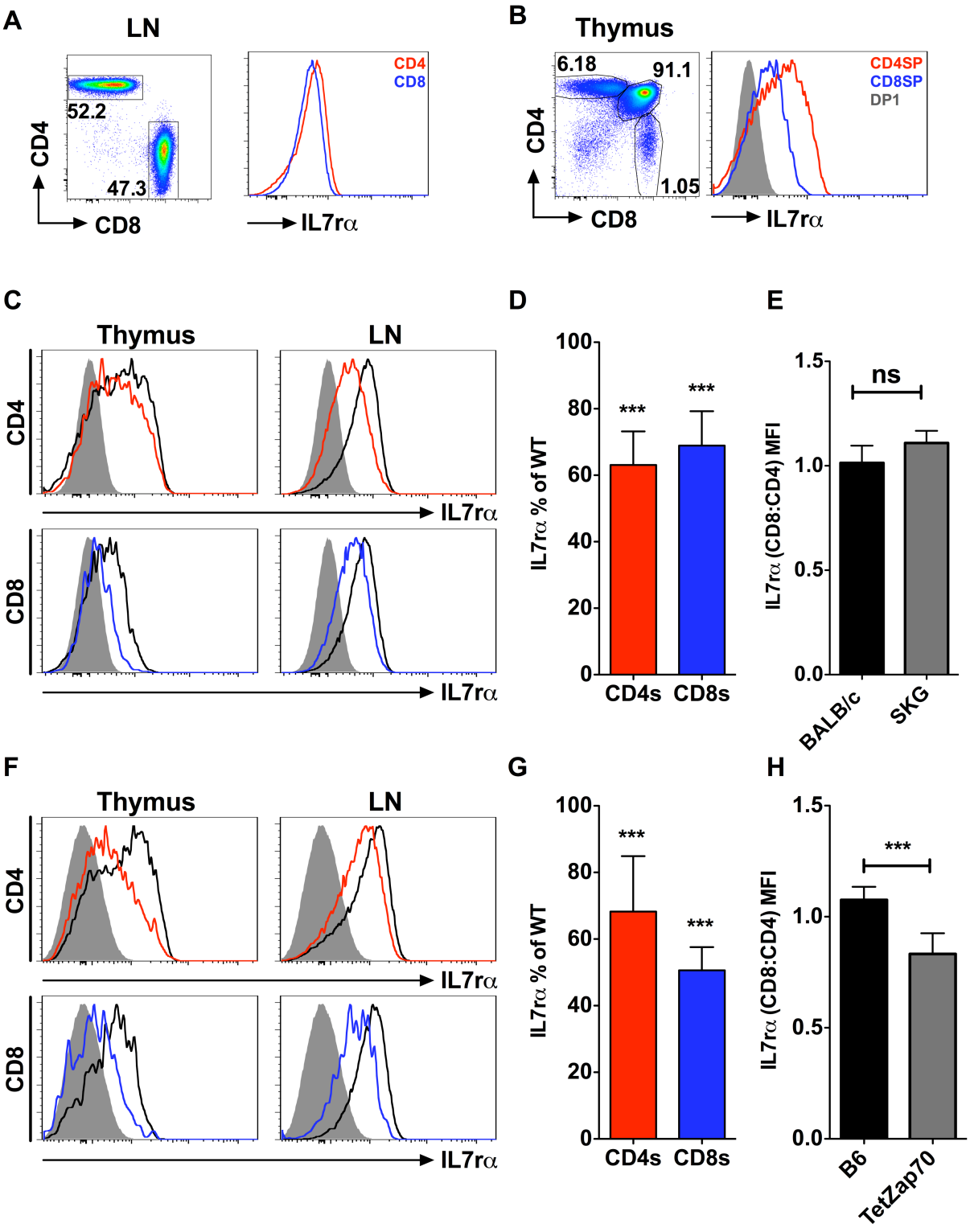

A
LN
52.2
47.3
CD4
CD8
CD4
CD8
IL7rα
B
Thymus
6.18
91.1
1.05
CD4
CD8
CD4SP
CD8SP
DP1
IL7rα
C
Thymus
LN
CD4
CD8
IL7rα
IL7rα
D
IL7rα % of WT
100
80
60
40
20
0
***
***
CD4s
CD8s
E
IL7rα (CD8:CD4) MFI
1.5
1.0
0.5
0.0
ns
BALB/c
SKG
F
Thymus
LN
CD4
CD8
IL7rα
IL7rα
G
IL7rα % of WT
100
80
60
40
20
0
***
***
CD4s
CD8s
H
IL7rα (CD8:CD4) MFI
1.5
1.0
0.5
0.0
***
B6
TetZap70

Figure 5.2 MHC-I restricted F5 *Rag1*$^{-/-}$ transgenic thymocytes receive a weak positive selection signal and show a defect in IL7rα re-expression

(**A**-**B**, **F**) LNs from B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice were analysed for the expression of CD4, CD8, TCR, CD5, CD25, CD44 and IL7rα by flow cytometry. (**A**) Density plots show CD4 against CD8 expression on naïve T-cells (CD25^{lo}CD44^{lo}TCR^{hi}CD5^{hi}). (**B**) Histograms shows CD5 expression on CD8 SPs from OT-I *Rag1*$^{-/-}$ (red line), F5 *Rag1*$^{-/-}$ (blue line) and B6 controls (black dotted line) (gating is shown in (**A**)). (**C**-**F**) Thymi from B6, F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice were analysed for the expression of CD4, CD8, TCR, CD5, CD69 and IL7rα by flow cytometry. (**C**) Density plots show CD4 against CD8 expression on live thymocytes and CD5 against TCR expression on DP thymocytes. (**E**) Histogram shows CD69 expression on TCR^{hi}CD5^{hi} DP thymocytes. (**F**) Histograms show IL7rα expression in DP2 (top row), DP3 (second row) and CD8 SP (third row) gated thymocytes, and CD8 SP LNT (bottom row) populations from B6 (dotted black line), F5 *Rag1*$^{-/-}$ (blue line) and OT-I *Rag1*$^{-/-}$ (red line) mice. B6 DP1 thymocytes (grey fill) are shown as a negative control. (**G**) DP2, DP3 and CD8 SP populations were sorted to high purity (≥95%) from OT-I *Rag1*$^{-/-}$ and F5 *Rag1*$^{-/-}$ mice. Cellular mRNA was purified and analysed by RT-qPCR. Bar chart shows the mean expression of IL7r mRNA relative to Hprt1. Analysis was performed in triplicate wells and error bars represent the sd. Data is representative of ≥3 independent experiments.

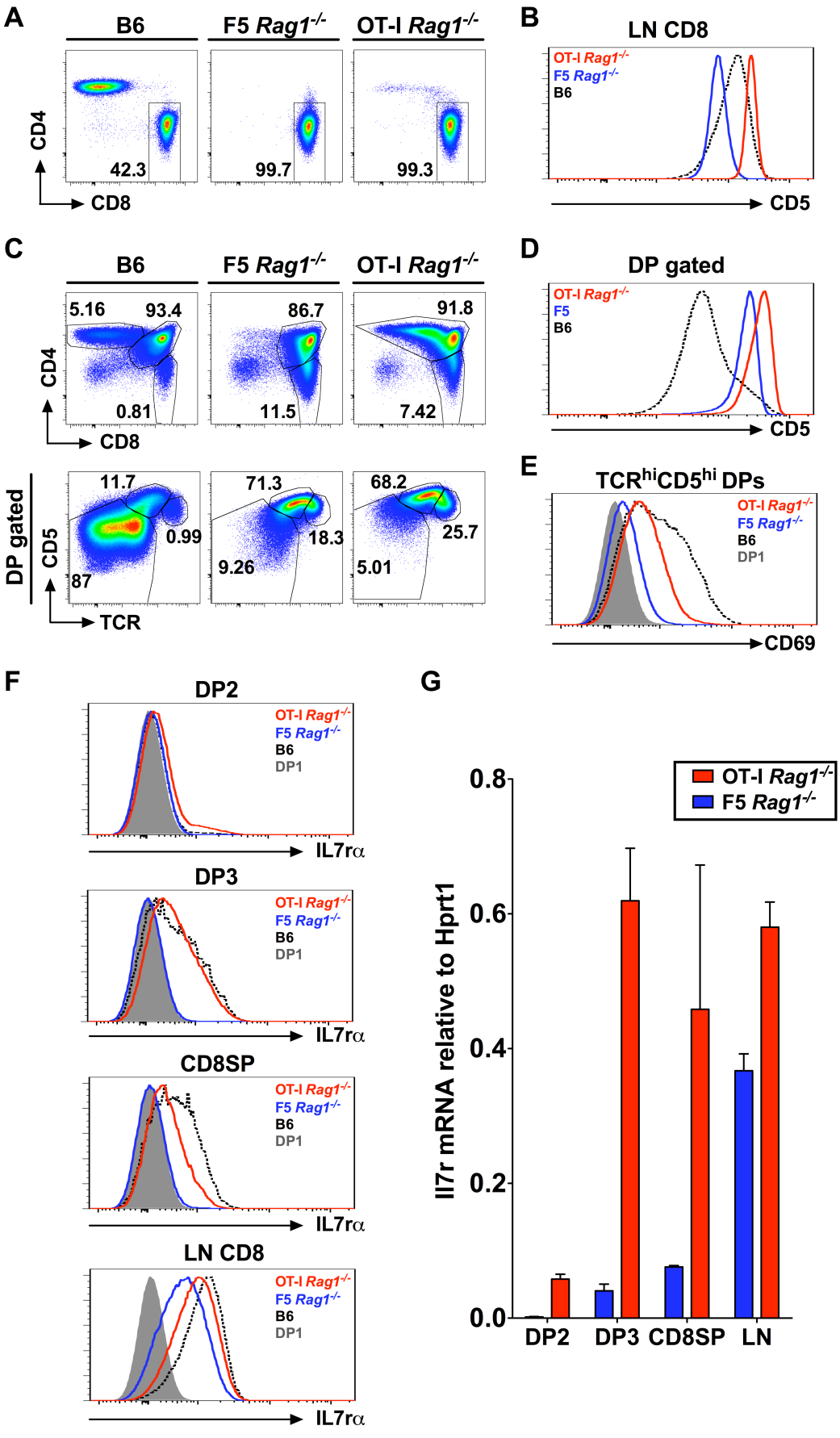

A
B6
F5 Rag1-/-
OT-I Rag1-/-
CD4
CD8
42.3
99.7
99.3
B
LN CD8
OT-I Rag1-/-
F5 Rag1-/-
B6
CD5
C
B6
F5 Rag1-/-
OT-I Rag1-/-
5.16
93.4
0.81
86.7
11.5
91.8
7.42
CD4
CD8
DP gated
CD5
TCR
11.7
0.99
87
71.3
18.3
9.26
68.2
25.7
5.01
D
DP gated
OT-I Rag1-/-
F5
B6
CD5
E
TCRhiCD5hi DPs
OT-I Rag1-/-
F5 Rag1-/-
B6
DP1
CD69
F
DP2
DP3
CD8SP
LN CD8
OT-I Rag1-/-
F5 Rag1-/-
B6
DP1
IL7rα
G
OT-I Rag1-/-
F5 Rag1-/-
Il7r mRNA relative to Hprt1
0.0
0.2
0.4
0.6
0.8
DP2
DP3
CD8SP
LN

Figure 5.3 The defect in IL7rα re-expression in F5 *Rag1*$^{-/-}$ mice correlates with reduced Runx mRNA expression

DP2, DP3 and CD8 SP thymocytes were sorted to high purity (≥95%) or total LN cells were isolated from F5 *Rag1*$^{-/-}$ and OT-I *Rag1*$^{-/-}$ mice. Cellular mRNA was purified and analysed by RT-qPCR. Bar charts show the mean expression of (**A**) Foxo1, (**B**) Gabpb, (**C**) Runx1 and (**D**) Runx3 relative to Hprt1. Analysis was performed in triplicate wells and error bars represent the sd. Data is representative of ≥3 independent experiments.

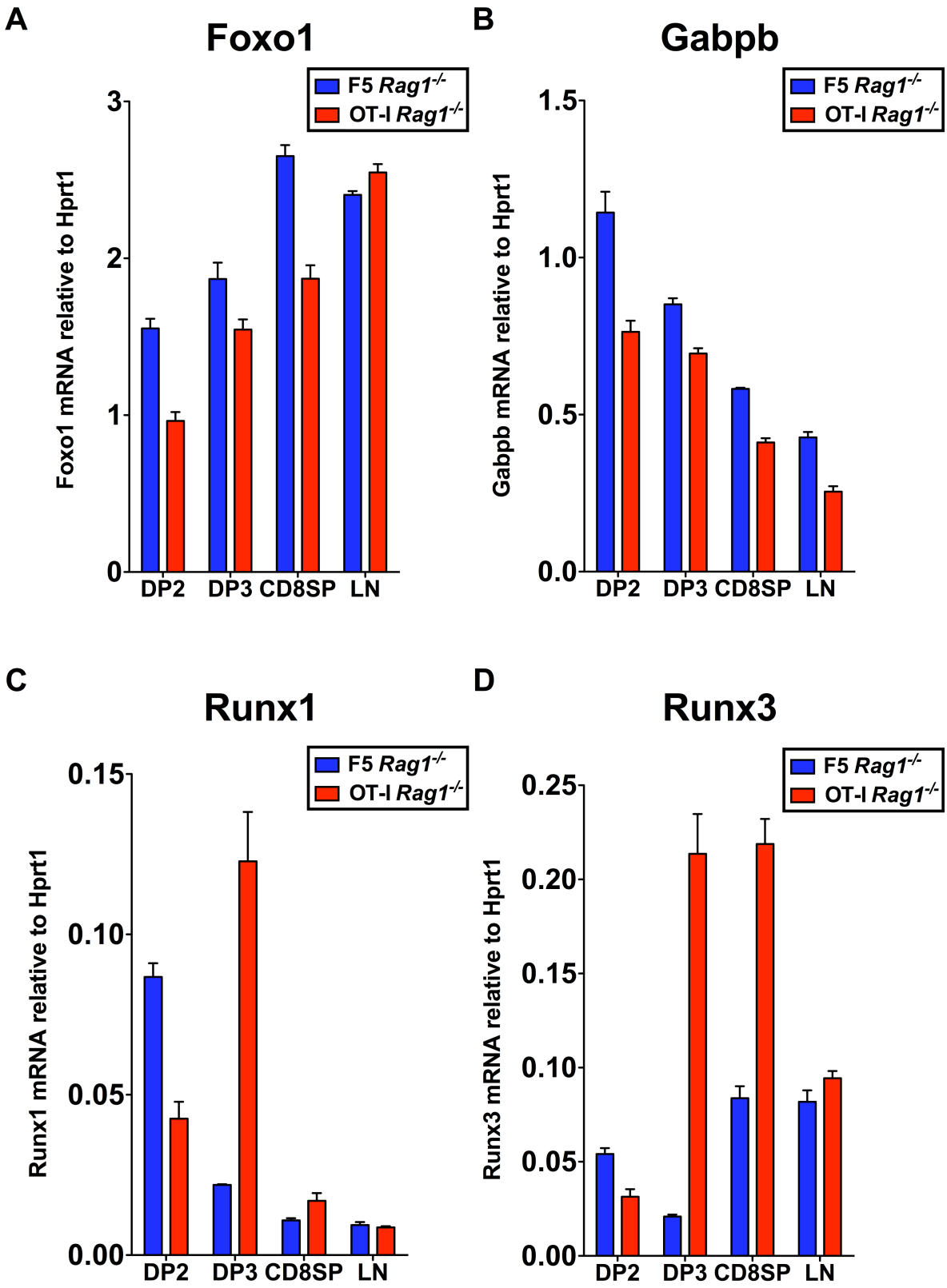

A
Foxo1
F5 Rag1-/-
OT-I Rag1-/-
Foxo1 mRNA relative to Hprt1
3
2
1
0
DP2
DP3
CD8SP
LN
B
Gabpb
F5 Rag1-/-
OT-I Rag1-/-
Gabpb mRNA relative to Hprt1
1.5
1.0
0.5
0.0
DP2
DP3
CD8SP
LN
C
Runx1
F5 Rag1-/-
OT-I Rag1-/-
Runx1 mRNA relative to Hprt1
0.15
0.10
0.05
0.00
DP2
DP3
CD8SP
LN
D
Runx3
F5 Rag1-/-
OT-I Rag1-/-
Runx3 mRNA relative to Hprt1
0.25
0.20
0.15
0.10
0.05
0.00
DP2
DP3
CD8SP
LN

Figure 5.4 Peripheral IL7rα expression is tuned by IL7 signals and not TCR signals in F5 mice

Total LN lymphocytes were harvested from F5 *Rag1*$^{-/-}$ CD45.1^{+} mice and 5x10^{6} cells were parked in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag1*$^{-/-}$, F5 *Rag1*$^{-/-}$ or B6 hosts (all CD45.2^{+}) for three days. Host LNs were then analysed by flow cytometry for the expression of CD4, CD8, TCR, CD5, CD25, CD44 and IL7rα. (**A**) Histograms show CD25 against CD44 on donor CD45.1^{+}TCR^{+} gated cells. (**B**) Histograms show CD5 expression on donor CD45.1^{+}TCR^{+} gated cells transferred to F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ (blue line, left), *Rag1*$^{-/-}$ (blue line, middle), B6 (blue line, right) and F5 *Rag1*$^{-/-}$ (grey fill) mice. (**C**) Histograms show IL7rα expression on donor CD45.1^{+}TCR^{+} gated cells transferred to F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ (blue line, left), *Rag1*$^{-/-}$ (blue line, middle), B6 (blue line, right) and F5 *Rag1*$^{-/-}$ (grey fill) mice, in addition to host B6 CD8 SP controls (black dotted line). (**D**) Total LN lymphocytes were harvested from F5 CD45.1^{+} mice and parked in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag1*$^{-/-}$, F5 *Rag1*$^{-/-}$ or B6 hosts (all CD45.2^{+}) for three days. Lymphocytes were then re-isolated from host LNs and treated with 10ng/ml IL7 (blue line) or left untreated (grey fill) at 37ºC for 30 minutes. Cells were immediately fixed and permeablised, before intracellular pSTAT5 expression was examined by flow cytometry. Histograms show pSTAT5 expression on CD45.1^{+} gated cells. Data are representative of ≥3 independent experiments.

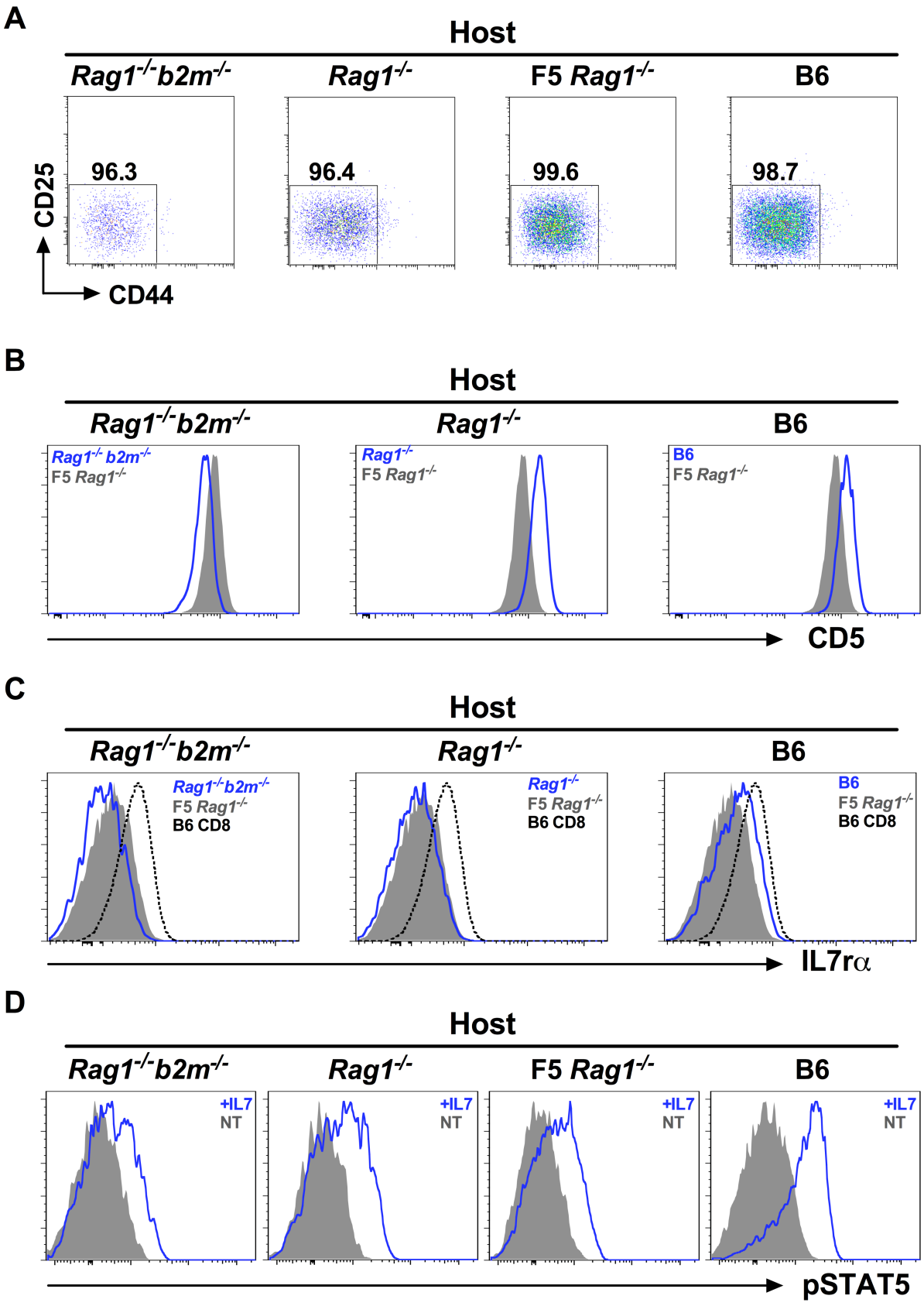

A
Host
Rag1-/-b2m-/-
Rag1-/-
F5 Rag1-/-
B6
96.3
96.4
99.6
98.7
CD25
CD44
B
Host
Rag1-/-b2m-/-
Rag1-/-
B6
Rag1-/- b2m-/-
F5 Rag1-/-
Rag1-/-
F5 Rag1-/-
B6
F5 Rag1-/-
CD5
C
Host
Rag1-/-b2m-/-
Rag1-/-
B6
Rag1-/-b2m-/-
F5 Rag1-/-
B6 CD8
Rag1-/-
F5 Rag1-/-
B6 CD8
B6
F5 Rag1-/-
B6 CD8
IL7rα
D
Host
Rag1-/-b2m-/-
Rag1-/-
F5 Rag1-/-
B6
+IL7
NT
+IL7
NT
+IL7
NT
+IL7
NT
pSTAT5

Figure 5.5 Peripheral IL7rα expression is tuned by IL7 signals and not TCR signals in OT-I mice

Total LN lymphocytes were harvested from OT-I *Rag1*$^{-/-}$ CD45.2^{+} mice and 5x10^{6} cells were parked in *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+}, *Rag2*$^{-/-}$ CD45.1^{+} or B6 CD45.1^{+} hosts for three days. Host LNs or control OT-I *Rag1*$^{-/-}$ LNs were then analysed by flow cytometry for the expression of CD4, CD8, TCR, CD5, CD25, CD44 and IL7rα. (**A**) Histograms show CD25 against CD44 on donor CD45.1^{+}TCR^{+} gated cells or OT-I *Rag1*$^{-/-}$ controls. (**B**) Histograms show CD5 expression on donor CD45.2^{+}TCR^{+} gated cells transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$ (red line, left), *Rag2*$^{-/-}$ (red line, middle), B6 (red line, right) or identically gated non-transferred OT-I *Rag2*$^{-/-}$ controls (grey fill). (**C**) Histograms show IL7rα expression on donor CD45.2^{+}TCR^{+} gated cells transferred to *Rag1*$^{-/-}$ *b2m*$^{-/-}$ (red line, left), *Rag2*$^{-/-}$ (red line, middle), B6 (red line, right) or identically gated non-transferred OT-I *Rag1*$^{-/-}$ controls (grey fill), in addition to host B6 CD8 SP controls (black dotted line). (**D**) Total LN lymphocytes were harvested from OT-I *Rag1*$^{-/-}$ CD45.2^{+} mice and parked in *Rag1*$^{-/-}$ *b2m*$^{-/-}$, *Rag2*$^{-/-}$ or B6 hosts (all CD45.2^{+}) for three days. Lymphocytes were then re-isolated from host LNs or isolated from intact OT-I *Rag1*$^{-/-}$ controls and treated with 10ng/ml IL7 (red line) or left untreated (grey fill) at 37ºC for 30 minutes. Cells were immediately fixed and permeablised, before intracellular pSTAT5 expression was examined by flow cytometry. Histograms show pSTAT5 expression on CD45.1^{+} gated cells. Data are representative of ≥3 independent experiments.

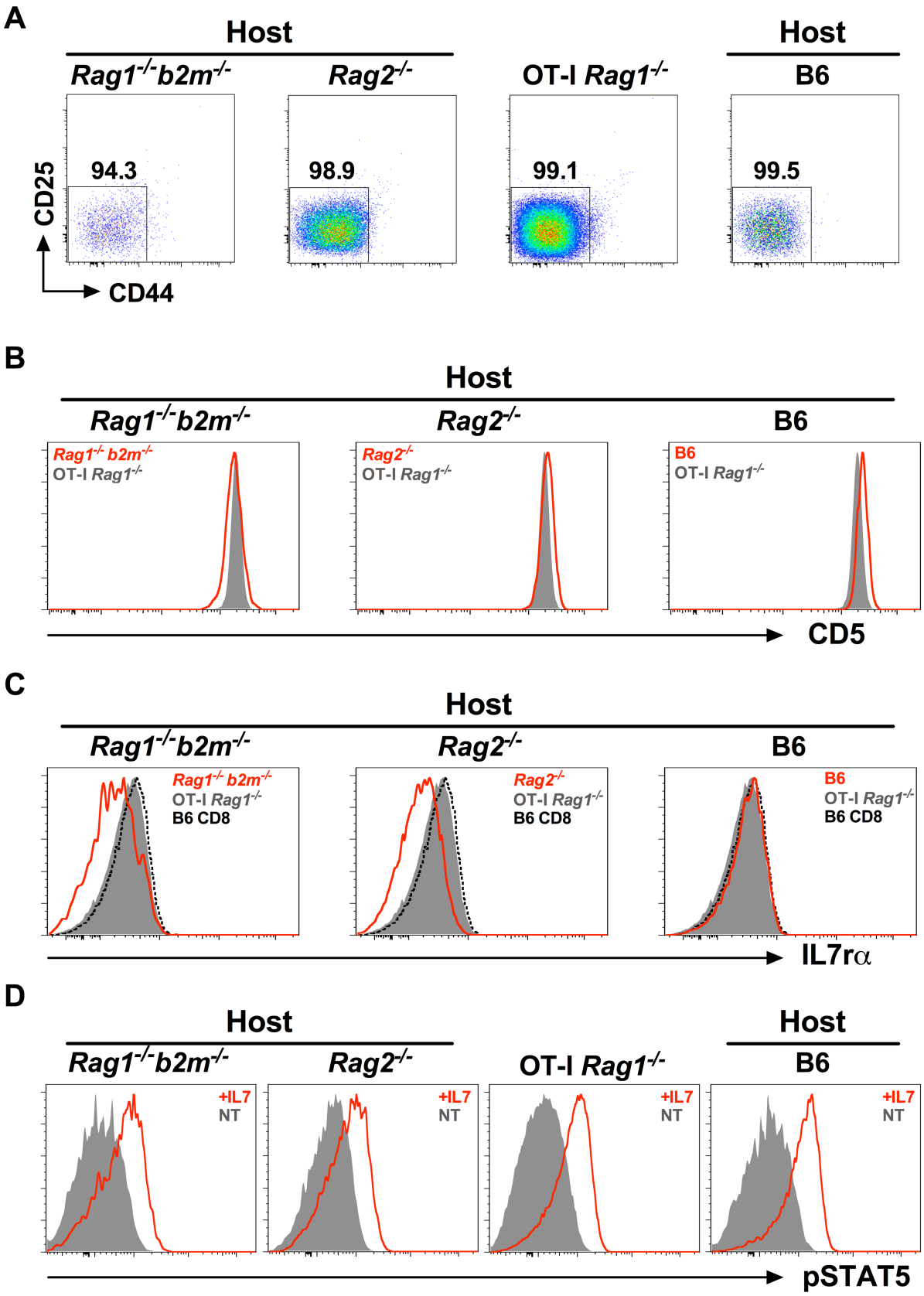
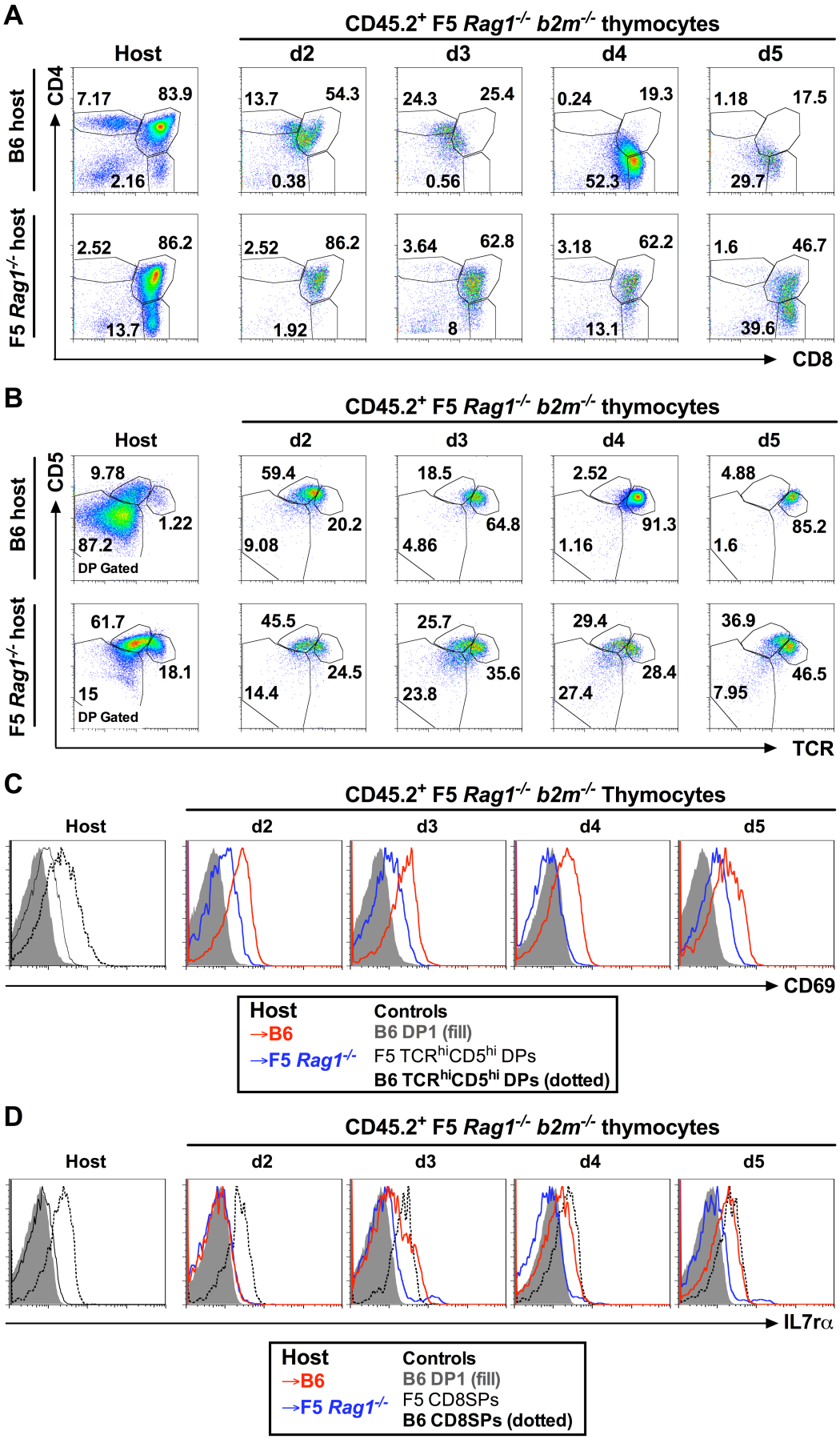

A
Host
Host
Rag1-/-b2m-/-
Rag2-/-
OT-I Rag1-/-
B6
94.3
98.9
99.1
99.5
CD25
CD44
B
Host
Rag1-/-b2m-/-
Rag2-/-
B6
Rag1-/- b2m-/-
OT-I Rag1-/-
Rag2-/-
OT-I Rag1-/-
B6
OT-I Rag1-/-
CD5
C
Host
Rag1-/-b2m-/-
Rag2-/-
B6
Rag1-/- b2m-/-
OT-I Rag1-/-
B6 CD8
Rag2-/-
OT-I Rag1-/-
B6 CD8
B6
OT-I Rag1-/-
B6 CD8
IL7rα
D
Host
Host
Rag1-/-b2m-/-
Rag2-/-
OT-I Rag1-/-
B6
+IL7
NT
+IL7
NT
+IL7
NT
+IL7
NT
pSTAT5

Figure 5.6 Augmentation of a weak positive selection signal restores IL7rα expression levels and kinetics

F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ CD45.2^{+} thymocytes (1-2x10^{6}) were intrathymically transferred to B6 CD45.1^{+}, or F5 CD45.1^{+} hosts. Host and donor thymocytes were analysed by flow cytometry 2-5 days (d2-5) following transfer. (**A**) Density plots show CD4 against CD8 expression on total host (CD45.1^{+}) or CD45.2^{+} donor thymocytes. (**B**) Density plots show CD5 against TCR expression on DP gated host (CD45.1^{+}) or total CD45.2^{+} donor thymocytes. (**C**) Histograms show CD69 expression on CD45.2^{+} donor thymocytes transferred to B6 (red line) or F5 (blue line) hosts, in addition to B6 DP1 (grey fill), B6 TCR^{hi}CD5^{hi} DP (dotted black line) and F5 TCR^{hi}CD5^{hi} DP (black line) host mouse controls (all CD45.1^{+}). (**D**) Histograms show IL7rα expression on CD45.2^{+} donor thymocytes transferred to B6 (red line) or F5 *Rag1*$^{-/-}$ (blue line) hosts, in addition to B6 DP1 (grey fill), B6 CD8 SP (dotted black line) and F5 *Rag1*$^{-/-}$ CD8 SP (black line) host mouse controls (all CD45.1^{+}). Data are representative of ≥2 replicate mice per timepoint.


A
CD45.2+ F5 Rag1-/- b2m-/- thymocytes
Host
d2
d3
d4
d5
B6 host
F5 Rag1-/- host
CD4
CD8
7.17
83.9
2.16
13.7
54.3
0.38
24.3
25.4
0.56
0.24
19.3
52.3
1.18
17.5
29.7
2.52
86.2
13.7
2.52
86.2
1.92
3.64
62.8
8
3.18
62.2
13.1
1.6
46.7
39.6
B
CD45.2+ F5 Rag1-/- b2m-/- thymocytes
Host
d2
d3
d4
d5
B6 host
F5 Rag1-/- host
CD5
TCR
9.78
1.22
87.2
DP Gated
59.4
20.2
9.08
18.5
64.8
4.86
2.52
91.3
1.16
4.88
85.2
1.6
61.7
18.1
15
DP Gated
45.5
24.5
14.4
25.7
35.6
23.8
29.4
28.4
27.4
36.9
46.5
7.95
C
CD45.2+ F5 Rag1-/- b2m-/- Thymocytes
Host
d2
d3
d4
d5
CD69
Host
→B6
→F5 Rag1-/-
Controls
B6 DP1 (fill)
F5 TCRhiCD5hi DPs
B6 TCRhiCD5hi DPs (dotted)
D
CD45.2+ F5 Rag1-/- b2m-/- thymocytes
Host
d2
d3
d4
d5
IL7rα
Host
→B6
→F5 Rag1-/-
Controls
B6 DP1 (fill)
F5 CD8SPs
B6 CD8SPs (dotted)

Chapter 6

Final Discussion

The overall aim of this thesis was to study the kinetics of positive selection and the CD4/CD8 lineage decision. Furthermore we investigated whether this process impacted on the future survival potential of naïve T-cells. We first sought to examine the kinetics of the CD4 versus CD8 lineage decision, and also to characterise the phenotypic changes associated with this event. This allowed us to gain a better understanding of how a positively selecting T-cell can interpret signals from either MHC-II or MHC-I. In the final results chapter we investigated a novel concept, that the nature of the positive selection may influence the future homeostatic survival capacity of a T-cell. The data presented in this thesis supports a view that CD4 and CD8 lineage commitment occurs with temporal distinction following the initiation of positive selection. Intriguingly, the CD4 and CD8 lineages were also found to develop from phenotypically distinct DP precursor populations. We provide evidence for differences in TCR signalling sensitivity between these populations, thus leading us to propose a model whereby the temporal regulation of thymic development allows for interpretation of the lineage commitment signal at distinct signalling thresholds. Finally our results suggest that positive selection TCR signalling does not merely instruct the lineage commitment decision but also determines the IL7rα expression levels in the periphery. Thus by implication, the positive

selection signal affects the future capacity of a mature T-cell to survive in the peripheral lymphoid organs.

6.1 The positive selection signal as a developmental process

Following restoration of positive selection, CD4 lineage commitment could occur as early as two days, whereas we saw no CD8 lineage cells until four days after positive selection was initiated (**Figure 3.2**, **Figure 3.8**). This finding represents a conceptual step forward with regards to our understanding of the CD4/CD8 lineage decision. Lucas and Germain first reported that positive selection and lineage commitment were temporally segregated events (Lucas & Germain, 1996). The TetZap70 inducible system enabled us to examine the kinetics of positive selection with improved resolution, allowing us to identify that the CD4 and CD8 lineage commitment events are also temporally distinct. Furthermore, we identified three temporally distinct DP populations based on the expression of TCR and CD5, which had different developmental potential. The DP1 population was present in TetZap70^{off} mice that are Zap70-deficient, and thus we conclude it was likely to contain the DP cells yet to receive a positive selection signal (**Figure 3.4**). In contrast the DP2 population contained the direct precursors of the CD4 lineage, and indirect precursors of the CD8 lineage. DP2 cells destined to become CD8 SPs had to first transit via the DP3 population (**Figure 3.8**, summarised in **Figure 3.12**). We suggest that subdividing the DP population into DP1-3 subpopulations permits finer resolution of developmentally intermediate populations in WT mice, compared

to the commonly employed strategy of gating based on subtle differences in coreceptor expression levels (Lucas & Germain, 1996) (reviewed in (Singer *et al.*, 2008)). Hence, our results may warrant a re-examination of mice reported previously to have defects in positive selection. Examination of the DP1-3 phenotype of such mice may give new insight into the nature of these developmental abnormalities.

We further identified that WT selecting thymocytes progressively upregulate Zap70 and are likely to tune expression of TCR and CD5, thus modulating their TCR signalling sensitivity throughout development (**Figure 3.2**, **Figure 4.1**, **Figure 4.2**). This may allow thymocytes to interpret MHC-II-mediated strong/consistent signals at an earlier time point, as these signals will require less TCR signalling sensitivity. In contrast, cells interpret a weak/intermittent MHC-I-mediated signal at a later time, when TCR signalling sensitivity is increased. Thus we hypothesise that the timing of the lineage commitment signal is critical to match MHC-specificity to lineage choice (summarised in **Figure 4.10**). Our model posits that positive selection resembles a permissive signalling threshold mechanism: a developmental signalling network found commonly in eukaryotic lineage specification. Such a mechanism is typified by a cells ability to make only one developmental choice within a particular temporal window (reviewed in (Freeman & Gurdon, 2002). Hence positive selection represents two closely linked permissive induction decisions. Early induction of TCR signalling will result in CD4 lineage commitment, whereas later induction results in CD8 commitment. Indeed the process whereby an identical

signalling pathway leads to different developmental outcomes depending on the temporal context has been reported in *Drosophila* eye development (Freeman, 1996), suggesting it may be a convergently evolved developmental strategy. It is likely that temporal networks operate to allow interpretation of the timing of developmental signalling. Thus it will be of interest to compare microarray analysis of DP2 and DP3 T-cells, to elucidate potential candidates playing such a role.

A unique feature of the CD4/CD8 lineage decision made by T-cells is that polyclonal TCRs have different avidities for spMHC ligand. Thus mechanisms have to ensure that wide-ranging avidities for spMHC do not lead to misinterpretation of the developmental signal. We hypothesise that redundancy exists to ensure that MHC-II versus MHC-I-mediated positive selection signal is divergent, and is largely imparted by the different properties of the CD4 or CD8 coreceptors. First, the cytoplasmic tail of CD4 binds to Lck with a higher affinity than CD8 (Wiest *et al.*, 1993), suggesting that MHC-II signals may be generally of a higher avidity. Second, following positive selection, the CD8 coreceptor is downregulated, suggesting that a CD8:spMHC-I signal is likely to be initially transient (reviewed in (Singer *et al.*, 2008)). Thus aspects of both quantitative and kinetic signalling models are likely to contribute to inherent differences in coreceptor-mediated recruitment of Lck.

Data presented herein provide further insight into how such strong/consistent versus weak/intermittent signals may be further differentiated. We find evidence for a positive feedback loop, whereby TCR signals directly upregulate Zap70

expression *in vitro* and *in vivo* (**Figure 4.8**, **Figure 4.9**), at the transcriptional level (**Figure 4.6**). Failure to upregulate Zap70 resulted in a developmental block particularly affecting the CD8 lineage (**Figure 4.4**) (Saini *et al.*, 2010)). We suggest that the positive autoregulation of Zap70 occurs as part of a feedforward loop, a common motif found in transcription factor networks. A feedforward loop commonly permits the resolution of developmental signals from inherent background noise common to biological signalling pathways (reviewed in (Alon, 2007). Thus regulating Zap70 directly downstream of the TCR may serve to further enhance differences in signalling, so that strong/consistent MHC-II signals increase TCR signalling sensitivity more rapidly then weak/intermittent MHC-I signals.

Paradoxically, the failure to upregulate Zap70 in TetZap70^{on} mice had only a minimal effect on the CD4 lineage (**Figure 3.1**). This may be explained if Zap70 expression is not rate-limiting in MHC-II-mediated TCR signal transduction. However we found that the CD4 lineage was enriched with cells expressing a relatively higher level of Zap70 compared to CD8 lineage cells, suggesting that this is not the case for all cells. It could be that high Zap70 expression early in positive selection promotes strong signalling associated with CD4 SP development, irrespective of MHC-restriction (**Figure 4.1**). In contrast, we showed that lower expression of Zap70 was associated with a developmental block at the DP3 stage of development (**Figure 4.4**). Critically, a developmental increase in TCR sensitivity may be required to progress beyond the DP3 stage, thus providing an explanation for why CD8 lineage cells appear to be more

affected by the loss of developmental Zap70 upregulation in TetZap70 thymocytes.

It is therefore possible that developmentally dysregulated Zap70 expression in TetZap70 mice leads to an incorrect lineage decision, resulting in mature MHC-mismatched T-cells. Whether this is the case could be revealed in mice where only MHC-II or MHC-I restricted T-cells are permitted to develop. Thus, future experiments in the lab will examine positive selection of TetZap70 thymocytes on MHC-II or MHC-I-deficient backgrounds. It may also be of interest to examine whether lineage misdirection occurs in mouse models where TCR or CD5 are perturbed during thymic selection. For example it has been reported in E3 ubiquitin-protein ligase Cbl (c-Cbl)-deficient mice that both TCR and CD5 are upregulated in DP thymocytes (Naramura *et al.*, 1998), but it is unknown whether this leads to the misinterpretation of the lineage commitment signal.

6.2 Impact of the positive selection signal on naïve T-cell homeostasis

Positive selection has been thought of as an isolated developmental event, functioning for the sole purpose of instructing MHC-II restricted cells to the CD4 lineage and MHC-I restricted cells to the CD8 lineage. However, we find evidence suggesting that the 'quality' of the positive selection will impact on the future homeostatic survival potential of T-cells. We found both in polyclonal mouse models with defects in TCR signalling, and in transgenic mouse models

with weak spMHC avidity, that IL7rα was suboptimally re-expressed following positive selection (**Figure 5.1**, **Figure 5.2**). When we intrathymically transferred F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ TCR transgenic T-cells to F5 *Rag1*$^{-/-}$ hosts, donor cells received a weak positive selection signal, but importantly there was no impact on the kinetics with which lineage commitment progressed (**Figure 5.6 A**-**B**, lower rows). Thus, signalling was sufficient to permit lineage commitment, but inadequate to allow for IL7rα re-expression. In contrast, improving the positive selection signalling strength by transferring F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ cells to B6 hosts increased both the positive selection signal strength as measured by CD69 expression, and also restored IL7rα expression (**Figure 5.6**). Such a mechanism would result in T-cells retaining a memory of their positive selection signalling strength.

TCR and IL7 signals have partially redundant function in naïve T-cell survival (Seddon & Zamoyska, 2002a). We have not only found evidence that the positive selection signal tunes IL7rα abundance, but also that Zap70 was expressed with lower abundance in F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes receiving a suboptimal positive selection signal (**Figure 4.9**). Hence the positive selection signal apparently affects both TCR and IL7rα signalling sensitivity in weakly signalled cells. Such a model is reminiscent of B-cell positive selection signalling (reviewed in (Cancro & Kearney, 2004)). Walmsley et al. have shown that B-cells deficient in Rac1 or Rac2 during positive selection have defective upregulation of BAFFR (Walmsley *et al.*, 2003). Furthermore, BCR signals are required to induce synthesis of the NFκB substrate p100, essential for BAFFR

signal transduction (Stadanlick *et al.*, 2008). It remains controversial whether TCR signals augment IL7rα signalling in mature T-cells, or indeed whether physiological differences in the positive selection signal strength affect mature BCR signal transduction.

An implication of our findings is that T-cell clones receiving a strong positive selection signal are more likely to survive in the periphery, as they will be able to outcompete cells for survival factors. Boursalian et al. reported that IL7rα was progressively upregulated over approximately two weeks following thymic egress, on both CD4 and CD8 naïve T-cells (Boursalian *et al.*, 2004). An alternative interpretation of these findings would be that IL7rα^{lo} cells were progressively lost over this period. Indeed we are currently investigating this hypothesis, by comparing the survival potential of WT IL7rα^{hi} and IL7rα^{lo} thymocytes transferred to congenic hosts. A further question arising from our data is why such a preference for strong spMHC recognition during thymic development would be beneficial to naïve T-cells? Such a mechanism would certainly be favourable in eliminating any T-cells that escape positive selection with defects in TCR structure or associated signalling machinery. However, it is not clear whether such cells exist in the periphery given the strong competition during positive selection in WT mice. A mechanism whereby IL7rα expression is lower on weakly selected cells could alternatively favour clonal diversity, as it may reduce intraclonal competition. For example, we found that by reducing intraclonal competition between selecting F5 *Rag1*$^{-/-}$ *b2m*$^{-/-}$ thymocytes, Zap70 and IL7rα expression was restored (**Figure 4.9**, **Figure 5.6**). However, TCR

transgenic mice are likely to represent an extreme case. Even in the low avidity F5 *Rag1*$^{-/-}$ mice, approximately 1x10^{7} cells can be sustained in the peripheral lymph nodes (Mamalaki *et al.*, 1993), approximately 20-25% of the total numbers residing in WT lymph nodes (**Figure 4.5**). Thus it is unclear whether the availability of spMHC would be rate limiting for intraclonal competition. Furthermore, T-cells are likely to be cross-reactive for identical self-peptides (reviewed in (Mason, 1998)), so such a mechanism may result in a loss of naïve T-cell repertoire diversity via outcompetition of weaker avidity clones.

An attractive hypothesis as to why T-cells with strong spMHC avidities are favoured is that spMHC recognition is also likely to be required during T-cell activation. The absence of self-recognition has been shown to impair the activation of both naïve and memory T-cells (Kassiotis *et al.*, 2002; Stefanova *et al.*, 2002). Furthermore, it has been shown that a single foreign peptide antigen displayed in the context of MHC (pMHC) interaction can elicit a calcium response in T-cells provided that additional TCR:spMHC interactions are aggregated into a signalling complex (Irvine *et al.*, 2002). Thus the continual refinement of the adaptive immune system through preferential loss of low avidity clones and continual input from thymic development may ensure a progressive accumulation of higher avidity clones, which are most likely to elicit an immune response. It will be interesting to investigate whether IL7rα^{hi} and IL7rα^{lo} naïve T-cells from WT polyclonal mice show a different propensity for activation, following an immune challenge.

6.3 Future perspectives

The results presented in this thesis expand on the existing knowledge of positive selection and CD4/CD8 lineage development. Furthermore, our results raise a number of questions about this process. For example, the CD4 SP population in TetZap70 mice is enriched with cells expressing a relatively high level of Zap70 compared to the CD8 SP population (**Figure 4.1 B**). It would be interesting to address whether MHC-II restricted cells with low levels of Zap70 are redirected to the CD8 lineage, and likewise whether MHC-I-restricted cells with high Zap70 expression are redirected to the CD4 lineage. Such a question could be addressed in a system where *b2m*$^{-/-}$ and MHC-II Δ/Δ hosts were reconstituted with TetZap70 BM. These BM chimeras could be induced with dox but would only permit the development of MHC-II or MHC-I restricted cells respectively, and thus would enable the identification of mismatched lineage development.

We have also identified that the branch point between CD4 and CD8 lineages occurs at the DP2 stage. Thus the DP2 population comprises both MHC-II and MHC-I-restricted thymocytes. It is not known whether there is homeostatic competition between MHC-II and MHC-I-restricted thymocytes undergoing positive selection. We hypothesise that if no cross-competition existed, the sum of the DP2 population frequencies in *b2m*$^{-/-}$ and MHC-II Δ/Δ mice should approximately equal the WT DP2 frequency. Interestingly, upon gross examination of the thymic phenotype, the DP2 frequencies appear to be larger than expected in *b2m*$^{-/-}$ and/or MHC-II Δ/Δ mice (**Figure 3.6 B**), suggesting that

the homeostasis of DP2 cells is indeed impacted by cells selecting to an alternative lineage. Due to the potentially complex population dynamics involved, a mathematical modelling approach could be used to identify whether interclonal competition exists between MHC-II and MHC-I-restricted DP2 thymocyte populations. Such an approach has been successful elsewhere to model the dynamics of homeostatic proliferation, revealing that a seemingly complex cellular process could be predicted using a relatively simple stochastic single-division model (Yates *et al.*, 2008). Mathematical modelling of DP subset homeostasis would be further aided by analysing the development of MHC-II and MHC-I-restricted cells independently, as would be possible using a BM chimeric system where *b2m*$^{-/-}$ and MHC-II Δ/Δ host mice were reconstituted with TetZap70 BM.

Finally, we find that different levels of Zap70 and IL7rα expression are apparently set by the positive selection signal. Furthermore the upregulation of Zap70 is critical for this process. For example, failure to upregulate Zap70 results in a specific defect in CD8 lineage development (**Figure 4.4**) (Saini *et al.*, 2010). Thus it is of interest to investigate the genetic elements controlling these processes. This could involve characterisation of chromatin modifications during positive selection, in addition to identification of transcription factors that directly bind the *Zap70* or *Il7r* loci. The eventual aim of such studies would be to generate mice with mutated genetic regulatory elements, to study their role during T-cell development and/or naïve T-cell differentiation.

6.4 Conclusions

The CD4/CD8 lineage decision employs sophisticated regulatory mechanisms to ensure the accurate coupling of MHC-restriction to lineage choice. This process has served as a model system in which to study haematopoietic differentiation. Our data represents a conceptual step forward from current models of CD4/CD8 development. We show that further to the temporal separation of positive selection and lineage commitment, development of CD4 and CD8 lineages is also temporally distinct. In addition, our results identify that kinetic changes in TCR signalling sensitivity underlie this developmental process, and may be crucial for the resolution of MHC-II versus MHC-I-mediated selection signals. Finally, we argue that the lineage commitment decision is not simply an isolated process, but rather confers differing survival capabilities to T-cells, based on the quality of the positive selection signal. This has far reaching consequences on the naïve T-cell repertoire, as not all T-cells surviving positive selection are equally likely to persist in the peripheral lymphoid organs.

References

Aaltonen, J., Bjorses, P., Perheentupa, J. & other authors (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nature Genetics* **17**, 399-403.

Acuto, O., Di Bartolo, V. & Michel, F. (2008). Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nature Reviews Immunology* **8**, 699-712.

Adachi, S., Amasaki, Y., Miyatake, S., Arai, N. & Iwata, M. (2000). Successive expression and activation of NFAT family members during thymocyte differentiation. *The Journal of Biological Chemistry* **275**, 14708-14716.

Adachi, S., Kuwata, T., Miyaike, M. & Iwata, M. (2001). Induction of CCR7 expression in thymocytes requires both ERK signal and Ca(2+) signal. *Biochemical and Biophysical Research Communications* **288**, 1188-1193.

Adoro, S., Erman, B., Sarafova, S. D., Van Laethem, F., Park, J. H., Feigenbaum, L. & Singer, A. (2008). Targeting CD4 coreceptor expression to postselection thymocytes reveals that CD4/CD8 lineage choice is neither error-prone nor stochastic. *The Journal of Immunology* **181**, 6975-6983.

Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R. & Weissman, I. L. (1997). Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell* **89**, 1033-1041.

Akashi, K., Kondo, M. & Weissman, I. L. (1998). Two distinct pathways of positive selection for thymocytes. *Proceedings of the National Academy of Sciences U S A* **95**, 2486-2491.

Akira, S. & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology* **4**, 499-511.

Alam, S. M., Travers, P. J., Wung, J. L., Nasholds, W., Redpath, S., Jameson, S. C. & Gascoigne, N. R. (1996). T-cell-receptor affinity and thymocyte positive selection. *Nature* **381**, 616-620.

Alarcon, B. & van Santen, H. M. (2010). Two receptors, two kinases, and T cell lineage determination. *Science Signaling* **3**, pe11.

Aliahmad, P., O'Flaherty, E., Han, P., Goularte, O. D., Wilkinson, B., Satake, M., Molkentin, J. D. & Kaye, J. (2004). TOX provides a link between calcineurin activation and CD8 lineage commitment. *The Journal of Experimental Medicine* **199**, 1089-1099.

Aliahmad, P. & Kaye, J. (2008). Development of all CD4 T lineages requires nuclear factor TOX. *The Journal of Experimental Medicine* **205**, 245-256.

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nature Reviews Genetics* **8**, 450-461.

Anderson, G., Owen, J. J., Moore, N. C. & Jenkinson, E. J. (1994). Characteristics of an in vitro system of thymocyte positive selection. *The Journal of Immunology* **153**, 1915-1920.

Anderson, G. & Jenkinson, E. J. (2001). Lymphostromal interactions in thymic development and function. *Nature Reviews Immunology* **1**, 31-40.

Anderson, G., Lane, P. J. & Jenkinson, E. J. (2007). Generating intrathymic microenvironments to establish T-cell tolerance. *Nature Reviews Immunology* **7**, 954-963.

Anderson, M. S., Venanzi, E. S., Klein, L. & other authors (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395-1401.

Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X. & Perlmutter, R. M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* **70**, 751-763.

Arpaia, E., Shahar, M., Dadi, H., Cohen, A. & Roifman, C. M. (1994). Defective T cell receptor signaling and CD8+ thymic selection in humans lacking zap-70 kinase. *Cell* **76**, 947-958.

Aspinall, R., Del Giudice, G., Effros, R. B., Grubeck-Loebenstein, B. & Sambhara, S. (2007). Challenges for vaccination in the elderly. *Immunity & Ageing* **4**, 9.

Au-Yeung, B. B., Deindl, S., Hsu, L. Y., Palacios, E. H., Levin, S. E., Kuriyan, J. & Weiss, A. (2009). The structure, regulation, and function of ZAP-70. *Immunological Reviews* **228**, 41-57.

Azzam, H. S., Grinberg, A., Lui, K., Shen, H., Shores, E. W. & Love, P. E. (1998). CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *The Journal of Experimental Medicine* **188**, 2301-2311.

Azzam, H. S., DeJarnette, J. B., Huang, K., Emmons, R., Park, C. S., Sommers, C. L., El-Khoury, D., Shores, E. W. & Love, P. E. (2001). Fine tuning of TCR signaling by CD5. *The Journal of Immunology* **166**, 5464-5472.

Baeuerle, P. A. & Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. *Annual Reviews of Immunology* **12**, 141-179.

Bain, G., Engel, I., Robanus Maandag, E. C. & other authors (1997). E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Molecular Cell Biology* **17**, 4782-4791.

Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunology and Cell Biology* **76**, 34-40.

Baron, C. & Penit, C. (1990). Study of the thymocyte cell cycle by bivariate analysis of incorporated bromodeoxyuridine and DNA content. *European Journal of Immunology* **20**, 1231-1236.

Barthlott, T., Kohler, H. & Eichmann, K. (1997). Asynchronous coreceptor downregulation after positive thymic selection: prolonged maintenance of the double positive state in CD8 lineage differentiation due to sustained biosynthesis of the CD4 coreceptor. *The Journal of Experimental Medicine* **185**, 357-362.

Basson, M. A., Bommhardt, U., Cole, M. S., Tso, J. Y. & Zamoyska, R. (1998). CD3 ligation on immature thymocytes generates antagonist-like signals appropriate for CD8 lineage commitment, independently of T cell receptor specificity. *The Journal of Experimental Medicine* **187**, 1249-1260.

Berzins, S. P., Boyd, R. L. & Miller, J. F. (1998). The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *The Journal of Experimental Medicine* **187**, 1839-1848.

Boehm, T. & Bleul, C. C. (2007). The evolutionary history of lymphoid organs. *Nature Immunology* **8**, 131-135.

Bogdan, C. (2001). Nitric oxide and the immune response. *Nature Immunology* **2**, 907-916.

Bommhardt, U., Cole, M. S., Tso, J. Y. & Zamoyska, R. (1997). Signals through CD8 or CD4 can induce commitment to the CD4 lineage in the thymus. *European Journal of Immunology* **27**, 1152-1163.

Bosselut, R., Feigenbaum, L., Sharrow, S. O. & Singer, A. (2001). Strength of signaling by CD4 and CD8 coreceptor tails determines the number but not the lineage direction of positively selected thymocytes. *Immunity* **14**, 483-494.

Boursalian, T. E., Golob, J., Soper, D. M., Cooper, C. J. & Fink, P. J. (2004). Continued maturation of thymic emigrants in the periphery. *Nature Immunology* **5**, 418-425.

Brack, C., Hirama, M., Lenhard-Schuller, R. & Tonegawa, S. (1978). A complete immunoglobulin gene is created by somatic recombination. *Cell* **15**, 1-14.

Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T. I., Yamashita, Y., Sharrow, S. O. & Singer, A. (2000). Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* **13**, 59-71.

Butcher, E. C. & Picker, L. J. (1996). Lymphocyte homing and homeostasis. *Science* **272**, 60-66.

Campbell, J. J., Pan, J. & Butcher, E. C. (1999). Cutting edge: developmental switches in chemokine responses during T cell maturation. *The Journal of Immunology* **163**, 2353-2357.

Cancro, M. P. & Kearney, J. F. (2004). B cell positive selection: road map to the primary repertoire? *The Journal of Immunology* **173**, 15-19.

Cancro, M. P. (2009). Signalling crosstalk in B cells: managing worth and need. *Nature Reviews Immunology* **9**, 657-661.

Canelles, M., Park, M. L., Schwartz, O. M. & Fowlkes, B. J. (2003). The influence of the thymic environment on the CD4-versus-CD8 T lineage decision. *Nature Immunology* **4**, 756-764.

Cantrell, D. A. (2002). Transgenic analysis of thymocyte signal transduction. *Nature Reviews Immunology* **2**, 20-27.

Cao, X., Shores, E. W., Hu-Li, J. & other authors (1995). Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity* **2**, 223-238.

Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. (1983). Precursors of T cell growth factor producing cells in the thymus: ontogeny, frequency, and quantitative recovery in a subpopulation of phenotypically mature thymocytes defined by monoclonal antibody GK-1.5. *The Journal of Experimental Medicine* **158**, 1654-1671.

Chan, A. C., Iwashima, M., Turck, C. W. & Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* **71**, 649-662.

Chan, A. C., Kadlecek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T. G. & Weiss, A. (1994a). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science* **264**, 1599-1601.

Chan, A. C., van Oers, N. S., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A. & Weiss, A. (1994b). Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *The Journal of Immunology* **152**, 4758-4766.

Chan, S., Correia-Neves, M., Dierich, A., Benoist, C. & Mathis, D. (1998). Visualization of CD4/CD8 T cell commitment. *The Journal of Experimental Medicine* **188**, 2321-2333.

Chan, S. H., Cosgrove, D., Waltzinger, C., Benoist, C. & Mathis, D. (1993). Another view of the selective model of thymocyte selection. *Cell* **73**, 225-236.

Chandok, M. R., Okoye, F. I., Ndejembi, M. P. & Farber, D. L. (2007). A biochemical signature for rapid recall of memory CD4 T cells. *The Journal of Immunology* **179**, 3689-3698.

Chien, Y. H., Gascoigne, N. R., Kavaler, J., Lee, N. E. & Davis, M. M. (1984). Somatic recombination in a murine T-cell receptor gene. *Nature* **309**, 322-326.

Cho, J. H., Kim, H. O., Surh, C. D. & Sprent, J. (2010). T Cell Receptor-Dependent Regulation of Lipid Rafts Controls Naive CD8(+) T Cell Homeostasis. *Immunity* **32**, 214-226.

Chong, M. M., Cornish, A. L., Darwiche, R. & other authors (2003). Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8+ T cell differentiation. *Immunity* **18**, 475-487.

Collins, A., Littman, D. R. & Taniuchi, I. (2009). RUNX proteins in transcription factor networks that regulate T-cell lineage choice. *Nature Reviews Immunology* **9**, 106-115.

Correia-Neves, M., Mathis, D. & Benoist, C. (2001). A molecular chart of thymocyte positive selection. *European Journal of Immunology* **31**, 2583-2592.

Costello, P. S., Walters, A. E., Mee, P. J., Turner, M., Reynolds, L. F., Prisco, A., Sarner, N., Zamoyska, R. & Tybulewicz, V. L. (1999). The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. *Proceedings of the National Academy of Sciences U S A* **96**, 3035-3040.

Dadi, H., Ke, S. & Roifman, C. M. (1994). Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor is dependent on protein tyrosine kinase activity. *Blood* **84**, 1579-1586.

Dadi, H. K. & Roifman, C. M. (1993). Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *Journal of Clinical Investigation* **92**, 1559-1563.

Daeron, M. (1997). Fc receptor biology. *Annual Reviews of Immunology* **15**, 203-234.

Dave, V. P., Allman, D., Keefe, R., Hardy, R. R. & Kappes, D. J. (1998a). HD mice: a novel mouse mutant with a specific defect in the generation of CD4(+) T cells. *Proceedings of the National Academy of Sciences U S A* **95**, 8187-8192.

Dave, V. P., Keefe, R., Berger, M. A., Drbal, K., Punt, J. A., Wiest, D. L., Alarcon, B. & Kappes, D. J. (1998b). Altered functional responsiveness of thymocyte subsets from CD3delta-deficient mice to TCR-CD3 engagement. *International Immunology* **10**, 1481-1490.

Davey, G. M., Schober, S. L., Endrizzi, B. T., Dutcher, A. K., Jameson, S. C. & Hogquist, K. A. (1998). Preselection thymocytes are more sensitive to T cell receptor stimulation than mature T cells. *The Journal of Experimental Medicine* **188**, 1867-1874.

Davis, C. B., Killeen, N., Crooks, M. E., Raulet, D. & Littman, D. R. (1993). Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* **73**, 237-247.

Deindl, S., Kadlecek, T. A., Brdicka, T., Cao, X., Weiss, A. & Kuriyan, J. (2007). Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. *Cell* **129**, 735-746.

Di Santo, J. P., Aifantis, I., Rosmaraki, E., Garcia, C., Feinberg, J., Fehling, H. J., Fischer, A., von Boehmer, H. & Rocha, B. (1999). The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early alpha/beta T cell development. *The Journal of Experimental Medicine* **189**, 563-574.

DiSanto, J. P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. (1995). Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proceedings of the National Academy of Sciences U S A* **92**, 377-381.

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L. & Stone, J. C. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nature Immunology* **1**, 317-321.

Doyle, C. & Strominger, J. L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330**, 256-259.

Effros, R. B. (1998). Replicative senescence in the immune system: impact of the Hayflick limit on T-cell function in the elderly. *American Journal of Human Genetics* **62**, 1003-1007.

Egawa, T., Tillman, R. E., Naoe, Y., Taniuchi, I. & Littman, D. R. (2007). The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *The Journal of Experimental Medicine* **204**, 1945-1957.

Egawa, T. & Littman, D. R. (2008). ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nature Immunology* **9**, 1131-1139.

Egerton, M., Scollay, R. & Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. *Proceedings of the National Academy of Sciences U S A* **87**, 2579-2582.

Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A. & Parslow, T. G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science* **264**, 1596-1599.

Erman, B., Alag, A. S., Dahle, O. & other authors (2006). Coreceptor signal strength regulates positive selection but does not determine CD4/CD8 lineage choice in a physiologic in vivo model. *The Journal of Immunology* **177**, 6613-6625.

Ernst, B., Lee, D. S., Chang, J. M., Sprent, J. & Surh, C. D. (1999). The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* **11**, 173-181.

Evans, S. E., Xu, Y., Tuvim, M. J. & Dickey, B. F. (2010). Inducible innate resistance of lung epithelium to infection. *Annual Reviews of Physiology* **72**, 413-435.

Favata, M. F., Horiuchi, K. Y., Manos, E. J. & other authors (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *The Journal of Biological Chemistry* **273**, 18623-18632.

Fehling, H. J., Krotkova, A., Saint-Ruf, C. & von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* **375**, 795-798.

Festenstein, R. & Kioussis, D. (2000). Locus control regions and epigenetic chromatin modifiers. *Current Opinion in Genetics and Development* **10**, 199-203.

Finkel, T. H., McDuffie, M., Kappler, J. W., Marrack, P. & Cambier, J. C. (1987). Both immature and mature T cells mobilize Ca2+ in response to antigen receptor crosslinking. *Nature* **330**, 179-181.

Fischer, A. (2007). Human primary immunodeficiency diseases. *Immunity* **27**, 835-845.

Foxwell, B. M., Beadling, C., Guschin, D., Kerr, I. & Cantrell, D. (1995). Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *European Journal of Immunology* **25**, 3041-3046.

Frank, M. M. & Fries, L. F. (1991). The role of complement in inflammation and phagocytosis. *Immunology Today* **12**, 322-326.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651-660.

Freeman, M. & Gurdon, J. B. (2002). Regulatory principles of developmental signaling. *Annual Review of Cell and Developmental Biology* **18**, 515-539.

Freitas, A. A. & Rocha, B. (2000). Population biology of lymphocytes: the flight for survival. *Annual Reviews of Immunology* **18**, 83-111.

Fruman, D. A., Snapper, S. B., Yballe, C. M., Davidson, L., Yu, J. Y., Alt, F. W. & Cantley, L. C. (1999). Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science* **283**, 393-397.

Fugmann, S. D., Lee, A. I., Shockett, P. E., Villey, I. J. & Schatz, D. G. (2000). The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annual Reviews of Immunology* **18**, 495-527.

Fujihashi, K., McGhee, J. R., Kweon, M. N., Cooper, M. D., Tonegawa, S., Takahashi, I., Hiroi, T., Mestecky, J. & Kiyono, H. (1996). gamma/delta T cell-deficient mice have impaired mucosal immunoglobulin A responses. *The Journal of Experimental Medicine* **183**, 1929-1935.

Fung-Leung, W. P., Schilham, M. W., Rahemtulla, A., Kundig, T. M., Vollenweider, M., Potter, J., van Ewijk, W. & Mak, T. W. (1991). CD8 is needed for development of cytotoxic T cells but not helper T cells. *Cell* **65**, 443-449.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871-1875.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nature Reviews Immunology* **3**, 710-720.

Ge, Q., Bai, A., Jones, B., Eisen, H. N. & Chen, J. (2004). Competition for self-peptide-MHC complexes and cytokines between naive and memory CD8+ T cells expressing the same or different T cell receptors. *Proceedings of the National Academy of Sciences U S A* **101**, 3041-3046.

Gelfand, E. W., Weinberg, K., Mazer, B. D., Kadlecek, T. A. & Weiss, A. (1995). Absence of ZAP-70 prevents signaling through the antigen receptor on peripheral blood T cells but not on thymocytes. *The Journal of Experimental Medicine* **182**, 1057-1065.

Germain, R. N. (2002). T-cell development and the CD4-CD8 lineage decision. *Nature Reviews Immunology* **2**, 309-322.

Godfrey, D. I., Kennedy, J., Suda, T. & Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *The Journal of Immunology* **150**, 4244-4252.

Godfrey, D. I., Kennedy, J., Mombaerts, P., Tonegawa, S. & Zlotnik, A. (1994). Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. *The Journal of Immunology* **152**, 4783-4792.

Godfrey, D. I. & Berzins, S. P. (2007). Control points in NKT-cell development. *Nature Reviews Immunology* **7**, 505-518.

Goldrath, A. W. & Bevan, M. J. (1999). Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity* **11**, 183-190.

Goldschneider, I., Komschlies, K. L. & Greiner, D. L. (1986). Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *The Journal of Experimental Medicine* **163**, 1-17.

Gordon, S. (2002). Pattern recognition receptors: doubling up for the innate immune response. *Cell* **111**, 927-930.

Gossen, M. & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences U S A* **89**, 5547-5551.

Grabstein, K. H., Waldschmidt, T. J., Finkelman, F. D., Hess, B. W., Alpert, A. R., Boiani, N. E., Namen, A. E. & Morrissey, P. J. (1993). Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *The Journal of Experimental Medicine* **178**, 257-264.

Grandjean, I., Duban, L., Bonney, E. A., Corcuff, E., Di Santo, J. P., Matzinger, P. & Lantz, O. (2003). Are major histocompatibility complex molecules involved in the survival of naive CD4+ T cells? *The Journal of Experimental Medicine* **198**, 1089-1102.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M. J., Hayday, A. C. & von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell* **75**, 283-294.

Grusby, M. J., Auchincloss, H., Jr., Lee, R., Johnson, R. S., Spencer, J. P., Zijlstra, M., Jaenisch, R., Papaioannou, V. E. & Glimcher, L. H. (1993). Mice lacking major histocompatibility complex class I and class II molecules. *Proceedings of the National Academy of Sciences U S A* **90**, 3913-3917.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *The Journal of Biological Chemistry* **260**, 3440-3450.

Guler, M. L., Gorham, J. D., Hsieh, C. S., Mackey, A. J., Steen, R. G., Dietrich, W. F. & Murphy, K. M. (1996). Genetic susceptibility to Leishmania: IL-12 responsiveness in TH1 cell development. *Science* **271**, 984-987.

Haks, M. C., Krimpenfort, P., Borst, J. & Kruisbeek, A. M. (1998). The CD3gamma chain is essential for development of both the TCRalphabeta and TCRgammadelta lineages. *EMBO Journal* **17**, 1871-1882.

Haks, M. C., Lefebvre, J. M., Lauritsen, J. P., Carleton, M., Rhodes, M., Miyazaki, T., Kappes, D. J. & Wiest, D. L. (2005). Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* **22**, 595-606.

Hamada, H., Garcia-Hernandez Mde, L., Reome, J. B., Misra, S. K., Strutt, T. M., McKinstry, K. K., Cooper, A. M., Swain, S. L. & Dutton, R. W. (2009). Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. *The Journal of Immunology* **182**, 3469-3481.

Harty, J. T. & Badovinac, V. P. (2008). Shaping and reshaping CD8+ T-cell memory. *Nature Reviews Immunology* **8**, 107-119.

Havran, W. L., Poenie, M., Kimura, J., Tsien, R., Weiss, A. & Allison, J. P. (1987). Expression and function of the CD3-antigen receptor on murine CD4+8+ thymocytes. *Nature* **330**, 170-173.

Hayday, A. C. (2000). [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annual Reviews of Immunology* **18**, 975-1026.

Hayes, S. M., Li, L. & Love, P. E. (2005). TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* **22**, 583-593.

Hayflick, L. & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Experimental Cell Research* **25**, 585-621.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Experimental Cell Research* **37**, 614-636.

He, X., Dave, V. P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B. A. & Kappes, D. J. (2005). The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* **433**, 826-833.

He, X., Park, K., Wang, H., Zhang, Y., Hua, X., Li, Y. & Kappes, D. J. (2008). CD4-CD8 Lineage Commitment Is Regulated by a Silencer Element at the ThPOK Transcription-Factor Locus. *Immunity* **28**, 346-358.

He, X., Park, K. & Kappes, D. J. (2010). The role of ThPOK in control of CD4/CD8 lineage commitment. *Annual Reviews of Immunology* **28**, 295-320.

Hedin, K. E., Appleby, M. W. & Clapham, D. E. (1995). Developmental regulation of TCR-CD3-dependent [Ca2+]i responses of individual normal and pp59fyn-deficient T lymphocytes. *Immunology* **84**, 183-192.

Hernandez-Hoyos, G., Sohn, S. J., Rothenberg, E. V. & Alberola-Ila, J. (2000). Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity* **12**, 313-322.

Hernandez-Hoyos, G., Anderson, M. K., Wang, C., Rothenberg, E. V. & Alberola-Ila, J. (2003). GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* **19**, 83-94

Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology* **8**, 15-25.

Hofmeister, R., Khaled, A. R., Benbernou, N., Rajnavolgyi, E., Muegge, K. & Durum, S. K. (1999). Interleukin-7: physiological roles and mechanisms of action. *Cytokine and Growth Factor Reviews* **10**, 41-60.

Hogan, P. G., Lewis, R. S. & Rao, A. (2010). Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annual Reviews of Immunology* **28**, 491-533.

Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. & Carbone, F. R. (1994). T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17-27.

Hogquist, K. A. (2001). Signal strength in thymic selection and lineage commitment. *Current Opinion in Immunology* **13**, 225-231.

Hogquist, K. A., Baldwin, T. A. & Jameson, S. C. (2005). Central tolerance: learning self-control in the thymus. *Nature Reviews Immunology* **5**, 772-782.

Holst, J., Wang, H., Eder, K. D. & other authors (2008). Scalable signaling mediated by T cell antigen receptor-CD3 ITAMs ensures effective negative selection and prevents autoimmunity. *Nature Immunology* **9**, 658-666.

Hori, S., Nomura, T. & Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-1061.

Hsu, L. Y., Tan, Y. X., Xiao, Z., Malissen, M. & Weiss, A. (2009). A hypomorphic allele of ZAP-70 reveals a distinct thymic threshold for autoimmune disease versus autoimmune reactivity. *The Journal of Experimental Medicine*.

Huesmann, M., Scott, B., Kisielow, P. & von Boehmer, H. (1991). Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell* **66**, 533-540.

Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A. L., Appella, E. & Engelhard, V. H. (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* **255**, 1261-1263.

Imada, K., Bloom, E. T., Nakajima, H., Horvath-Arcidiacono, J. A., Udy, G. B., Davey, H. W. & Leonard, W. J. (1998). Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *The Journal of Experimental Medicine* **188**, 2067-2074.

Irvine, D. J., Purbhoo, M. A., Krogsgaard, M. & Davis, M. M. (2002). Direct observation of ligand recognition by T cells. *Nature* **419**, 845-849.

Itano, A., Kioussis, D. & Robey, E. (1994). Stochastic component to development of class I major histocompatibility complex-specific T cells. *Proceedings of the National Academy of Sciences U S A* **91**, 220-224.

Itano, A. & Robey, E. (2000). Highly efficient selection of CD4 and CD8 lineage thymocytes supports an instructive model of lineage commitment. *Immunity* **12**, 383-389.

Iwashima, M., Irving, B. A., van Oers, N. S., Chan, A. C. & Weiss, A. (1994). Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* **263**, 1136-1139.

Jameson, S. C. (2002). Maintaining the norm: T-cell homeostasis. *Nature Reviews Immunology* **2**, 547-556.

Jiang, Q., Li, W. Q., Hofmeister, R. R., Young, H. A., Hodge, D. R., Keller, J. R., Khaled, A. R. & Durum, S. K. (2004). Distinct regions of the interleukin-7 receptor regulate different Bcl2 family members. *Molecular and Cellular Biology* **24**, 6501-6513.

Jiang, Q., Li, W. Q., Aiello, F. B., Mazzucchelli, R., Asefa, B., Khaled, A. R. & Durum, S. K. (2005). Cell biology of IL-7, a key lymphotrophin. *Cytokine and Growth Factor Reviews* **16**, 513-533.

Johnston, J. A., Bacon, C. M., Finbloom, D. S. & other authors (1995). Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. *Proceedings of the National Academy of Sciences U S A* **92**, 8705-8709.

Jones, M. E. & Zhuang, Y. (2007). Acquisition of a functional T cell receptor during T lymphocyte development is enforced by HEB and E2A transcription factors. *Immunity* **27**, 860-870.

Jones-Carson, J., Vazquez-Torres, A., van der Heyde, H. C., Warner, T., Wagner, R. D. & Balish, E. (1995). Gamma delta T cell-induced nitric oxide production enhances resistance to mucosal candidiasis. *Nature Medicine* **1**, 552-557.

Josefowicz, S. Z. & Rudensky, A. (2009). Control of regulatory T cell lineage commitment and maintenance. *Immunity* **30**, 616-625.

June, C. H. & Rabinovitch, P. S. (1990). Flow cytometric measurement of intracellular ionized calcium in single cells with indo-1 and fluo-3. *Methods in Cell Biology* **33**, 37-58.

Kadlecek, T. A., van Oers, N. S., Lefrancois, L., Olson, S., Finlay, D., Chu, D. H., Connolly, K., Killeen, N. & Weiss, A. (1998). Differential requirements for ZAP-70 in TCR signaling and T cell development. *The Journal of Immunology* **161**, 4688-4694.

Kaiser, D. (2001). Building a multicellular organism. *Annual Reviews of Genetics* **35**, 103-123.

Kappes, D. J. & He, X. (2005). CD4-CD8 lineage commitment: an inside view. *Nature Immunology* **6**, 761-766.

Kappler, J., Kubo, R., Haskins, K., Hannum, C., Marrack, P., Pigeon, M., McIntyre, B., Allison, J. & Trowbridge, I. (1983). The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. *Cell* **35**, 295-302.

Kappler, J. W., Roehm, N. & Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell* **49**, 273-280.

Kassiotis, G., Garcia, S., Simpson, E. & Stockinger, B. (2002). Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nature Immunology* **3**, 244-250.

Keefe, R., Dave, V., Allman, D., Wiest, D. & Kappes, D. J. (1999). Regulation of lineage commitment distinct from positive selection. *Science* **286**, 1149-1153.

Kelly, K. A. & Scollay, R. (1990). Analysis of recent thymic emigrants with subset- and maturity-related markers. *International Immunology* **2**, 419-425.

Kerdiles, Y. M., Beisner, D. R., Tinoco, R., Dejean, A. S., Castrillon, D. H., DePinho, R. A. & Hedrick, S. M. (2009). Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nature Immunology* **10**, 176-184.

Kieper, W. C. & Jameson, S. C. (1999). Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proceedings of the National Academy of Sciences U S A* **96**, 13306-13311.

Kieper, W. C., Tan, J. T., Bondi-Boyd, B., Gapin, L., Sprent, J., Ceredig, R. & Surh, C. D. (2002). Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. *The Journal of Experimental Medicine* **195**, 1533-1539.

Kim, C. H., Pelus, L. M., White, J. R. & Broxmeyer, H. E. (1998a). Differential chemotactic behavior of developing T cells in response to thymic chemokines. *Blood* **91**, 4434-4443.

Kim, K., Lee, C. K., Sayers, T. J., Muegge, K. & Durum, S. K. (1998b). The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *The Journal of Immunology* **160**, 5735-5741.

Kirberg, J., Berns, A. & von Boehmer, H. (1997). Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. *The Journal of Experimental Medicine* **186**, 1269-1275.

Kirkpatrick, J. A., Jr. & DiGeorge, A. M. (1968). Congenital absence of the thymus. *American Journal of Roentgenology, Radium Therapy and Nuclear Medicine* **103**, 32-37.

Kishimoto, H. & Sprent, J. (1997). Negative selection in the thymus includes semimature T cells. *The Journal of Experimental Medicine* **185**, 263-271.

Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* **350**, 423-426.

Kohu, K., Sato, T., Ohno, S. & other authors (2005). Overexpression of the Runx3 transcription factor increases the proportion of mature thymocytes of the CD8 single-positive lineage. *The Journal of Immunology* **174**, 2627-2636.

Koller, B. H., Marrack, P., Kappler, J. W. & Smithies, O. (1990). Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science* **248**, 1227-1230.

Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S. & Sugamura, K. (1994). Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science* **263**, 1453-1454.

Kong, G., Dalton, M., Wardenburg, J. B., Straus, D., Kurosaki, T. & Chan, A. C. (1996). Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. *Molecular Cell Biology* **16**, 5026-5035.

Koyasu, S., Clayton, L. K., Lerner, A., Heiken, H., Parkes, A. & Reinherz, E. L. (1997). Pre-TCR signaling components trigger transcriptional activation of a rearranged TCR alpha gene locus and silencing of the pre-TCR alpha locus: implications for intrathymic differentiation. *International Immunology* **9**, 1475-1480.

Kronenberg, M. (2005). Toward an understanding of NKT cell biology: progress and paradoxes. *Annual Reviews of Immunology* **23**, 877-900.

Kurobe, H., Liu, C., Ueno, T. & other authors (2006). CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* **24**, 165-177.

Labrecque, N., Whitfield, L. S., Obst, R., Waltzinger, C., Benoist, C. & Mathis, D. (2001). How much TCR does a T cell need? *Immunity* **15**, 71-82.

Labrie, S. J., Samson, J. E. & Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nature Reviews Microbiology* **8**, 317-327.

Laky, K. & Fowlkes, B. J. (2008). Notch signaling in CD4 and CD8 T cell development. *Current Opinion in Immunology* **20**, 197-202.

Lam, K. P., Kuhn, R. & Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* **90**, 1073-1083.

Lassila, O., Vainio, O. & Matzinger, P. (1988). Can B cells turn on virgin T cells? *Nature* **334**, 253-255.

Le Campion, A., Vasseur, F. & Penit, C. (2000). Regulation and kinetics of premigrant thymocyte expansion. *European Journal of Immunology* **30**, 738-746.

Lee, H. C., Shibata, H., Ogawa, S., Maki, K. & Ikuta, K. (2005). Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *The Journal of Immunology* **174**, 7800-7806.

Legname, G., Seddon, B., Lovatt, M. & other authors (2000). Inducible expression of a p56Lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. *Immunity* **12**, 537-546.

Lenschow, D. J., Walunas, T. L. & Bluestone, J. A. (1996). CD28/B7 system of T cell costimulation. *Annual Reviews of Immunology* **14**, 233-258.

Levelt, C. N., Ehrfeld, A. & Eichmann, K. (1993). Regulation of thymocyte development through CD3. I. Timepoint of ligation of CD3 epsilon determines clonal deletion or induction of developmental program. *The Journal of Experimental Medicine* **177**, 707-716.

Levy, S., Hannenhalli, S. & Workman, C. (2001). Enrichment of regulatory signals in conserved non-coding genomic sequence. *Bioinformatics* **17**, 871-877.

Levy, Y., Lacabaratz, C., Weiss, L. & other authors (2009). Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. *The Journal of Clinical Investigation* **119**, 997-1007.

Li, L., Sad, S., Kagi, D. & Mosmann, T. R. (1997). CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *The Journal of Immunology* **158**, 4152-4161.

Lin, J. X., Migone, T. S., Tsang, M. & other authors (1995). The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* **2**, 331-339.

Lio, C. W. & Hsieh, C. S. (2008). A two-step process for thymic regulatory T cell development. *Immunity* **28**, 100-111.

Liu, C. & Hermann, T. E. (1978). Characterization of ionomycin as a calcium ionophore. *The Journal of Biological Chemistry* **253**, 5892-5894.

Liu, C. P., Ueda, R., She, J. & other authors (1993). Abnormal T cell development in CD3-zeta-/- mutant mice and identification of a novel T cell population in the intestine. *EMBO Journal* **12**, 4863-4875.

Liu, X., Adams, A., Wildt, K. F., Aronow, B., Feigenbaum, L. & Bosselut, R. (2003). Restricting Zap70 expression to CD4+CD8+ thymocytes reveals a T cell receptor-dependent proofreading mechanism controlling the completion of positive selection. *The Journal of Experimental Medicine* **197**, 363-373.

Liu, X. & Bosselut, R. (2004). Duration of TCR signaling controls CD4-CD8 lineage differentiation in vivo. *Nature Immunology* **5**, 280-288.

Loots, G. G. & Ovcharenko, I. (2004). rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Research* **32**, W217-221.

Love, P. E., Lee, J. & Shores, E. W. (2000). Critical relationship between TCR signaling potential and TCR affinity during thymocyte selection. *The Journal of Immunology* **165**, 3080-3087.

Lowin, B., Hahne, M., Mattmann, C. & Tschopp, J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650-652.

Lucas, B., Vasseur, F. & Penit, C. (1993). Normal sequence of phenotypic transitions in one cohort of 5-bromo-2'-deoxyuridine-pulse-labeled thymocytes. Correlation with T cell receptor expression. *The Journal of Immunology* **151**, 4574-4582.

Lucas, B., Vasseur, F. & Penit, C. (1994). Production, selection, and maturation of thymocytes with high surface density of TCR. *The Journal of Immunology* **153**, 53-62.

Lucas, B. & Germain, R. N. (1996). Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation. *Immunity* **5**, 461-477.

Luik, R. M., Wang, B., Prakriya, M., Wu, M. M. & Lewis, R. S. (2008). Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454**, 538-542.

Lundberg, K. & Shortman, K. (1994). Small cortical thymocytes are subject to positive selection. *The Journal of Experimental Medicine* **179**, 1475-1483.

Lundberg, K., Heath, W., Kontgen, F., Carbone, F. R. & Shortman, K. (1995). Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4-8+TCRhi thymocytes. *The Journal of Experimental Medicine* **181**, 1643-1651.

Luo, Z. X. (2007). Transformation and diversification in early mammal evolution. *Nature* **450**, 1011-1019.

Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. *Nature Reviews Immunology* **5**, 472-484.

Mackall, C. L., Hakim, F. T. & Gress, R. E. (1997). T-cell regeneration: all repertoires are not created equal. *Immunology Today* **18**, 245-251.

Madden, D. R. (1995). The three-dimensional structure of peptide-MHC complexes. *Annual Reviews of Immunology* **13**, 587-622.

Madsen, L., Labrecque, N., Engberg, J., Dierich, A., Svejgaard, A., Benoist, C., Mathis, D. & Fugger, L. (1999). Mice lacking all conventional MHC class II genes. *Proceedings of the National Academy of Sciences U S A* **96**, 10338-10343.

Magnan, A., Di Bartolo, V., Mura, A. M. & other authors (2001). T cell development and T cell responses in mice with mutations affecting tyrosines 292 or 315 of the ZAP-70 protein tyrosine kinase. *The Journal of Experimental Medicine* **194**, 491-505.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E. & Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *EMBO Journal* **14**, 4641-4653.

Mamalaki, C., Elliott, J., Norton, T., Yannoutsos, N., Townsend, A. R., Chandler, P., Simpson, E. & Kioussis, D. (1993). Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Developmental Immunology* **3**, 159-174.

Mason, D. (1998). A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunology Today* **19**, 395-404.

Masuda, K., Kakugawa, K., Nakayama, T., Minato, N., Katsura, Y. & Kawamoto, H. (2007). T cell lineage determination precedes the initiation of TCR beta gene rearrangement. *The Journal of Immunology* **179**, 3699-3706.

Matechak, E. O., Killeen, N., Hedrick, S. M. & Fowlkes, B. J. (1996). MHC class II-specific T cells can develop in the CD8 lineage when CD4 is absent. *Immunity* **4**, 337-347.

Mathis, D. & Benoist, C. (2009). Aire. *Annual Reviews of Immunology* **27**, 287-312.

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L. & Cyster, J. G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**, 355-360.

Maurice, D., Hooper, J., Lang, G. & Weston, K. (2007). c-Myb regulates lineage choice in developing thymocytes via its target gene Gata3. *The EMBO Journal* **26**, 3629-3640.

Mazzucchelli, R. & Durum, S. K. (2007). Interleukin-7 receptor expression: intelligent design. *Nature Reviews Immunology* **7**, 144-154.

McCaffrey, P. G., Perrino, B. A., Soderling, T. R. & Rao, A. (1993). NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *The Journal of Biological Chemistry* **268**, 3747-3752.

McCaughtry, T. M., Wilken, M. S. & Hogquist, K. A. (2007). Thymic emigration revisited. *The Journal of Experimental Medicine* **204**, 2513-2520.

McCaughtry, T. M., Baldwin, T. A., Wilken, M. S. & Hogquist, K. A. (2008). Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *The Journal of Experimental Medicine* **205**, 2575-2584.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397.

Mertsching, E., Burdet, C. & Ceredig, R. (1995). IL-7 transgenic mice: analysis of the role of IL-7 in the differentiation of thymocytes in vivo and in vitro. *International Immunology* **7**, 401-414.

Miller, J. F. (1961). Immunological function of the thymus. *Lancet* **2**, 748-749.

Misslitz, A., Pabst, O., Hintzen, G., Ohl, L., Kremmer, E., Petrie, H. T. & Forster, R. (2004). Thymic T cell development and progenitor localization depend on CCR7. *The Journal of Experimental Medicine* **200**, 481-491.

Modlin, R. L. (1994). Th1-Th2 paradigm: insights from leprosy. *The Journal of Investigative Dermatology* **102**, 828-832.

Molina, T. J., Kishihara, K., Siderovski, D. P. & other authors (1992). Profound block in thymocyte development in mice lacking p56lck. *Nature* **357**, 161-164.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A. & other authors (1992a). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* **360**, 225-231.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. & Papaioannou, V. E. (1992b). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-877.

Mor, A. & Philips, M. R. (2006). Compartmentalized Ras/MAPK signaling. *Annual Reviews of Immunology* **24**, 771-800.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology* **136**, 2348-2357.

Mosmann, T. R. & Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Reviews of Immunology* **7**, 145-173.

Munitic, I., Williams, J. A., Yang, Y., Dong, B., Lucas, P. J., El Kassar, N., Gress, R. E. & Ashwell, J. D. (2004). Dynamic regulation of IL-7 receptor expression is required for normal thymopoiesis. *Blood* **104**, 4165-4172.

Murali-Krishna, K., Lau, L. L., Sambhara, S., Lemonnier, F., Altman, J. & Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377-1381.

Muroi, S., Naoe, Y., Miyamoto, C., Akiyama, K., Ikawa, T., Masuda, K., Kawamoto, H. & Taniuchi, I. (2008). Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. *Nature Immunology* **9**, 1113-1121.

Murphy, K. M., Heimberger, A. B. & Loh, D. Y. (1990). Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* **250**, 1720-1723.

Murray, R., Suda, T., Wrighton, N., Lee, F. & Zlotnik, A. (1989). IL-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *International Immunology* **1**, 526-531.

Murre, C. (2005). Helix-loop-helix proteins and lymphocyte development. *Nature Immunology* **6**, 1079-1086.

Nakajima, H., Liu, X. W., Wynshaw-Boris, A., Rosenthal, L. A., Imada, K., Finbloom, D. S., Hennighausen, L. & Leonard, W. J. (1997). An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. *Immunity* **7**, 691-701.

Nakano, H., Mori, S., Yonekawa, H., Nariuchi, H., Matsuzawa, A. & Kakiuchi, T. (1998). A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. *Blood* **91**, 2886-2895.

Namen, A. E., Lupton, S., Hjerrild, K. & other authors (1988a). Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* **333**, 571-573.

Namen, A. E., Schmierer, A. E., March, C. J., Overell, R. W., Park, L. S., Urdal, D. L. & Mochizuki, D. Y. (1988b). B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *The Journal of Experimental Medicine* **167**, 988-1002.

Naramura, M., Kole, H. K., Hu, R. J. & Gu, H. (1998). Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proceedings of the National Academy of Sciences U S A* **95**, 15547-15552.

Negishi, I., Motoyama, N., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C. & Loh, D. Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* **376**, 435-438.

Nika, K., Soldani, C., Salek, M. & other authors (2010). Constitutively Active Lck Kinase in T Cells Drives Antigen Receptor Signal Transduction. *Immunity*.

Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X. & Leonard, W. J. (1993). Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science* **262**, 1877-1880.

Nolan, T., Hands, R. E. & Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature Protocols* **1**, 1559-1582.

Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H. & Littman, D. R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81.

Nosaka, T., van Deursen, J. M., Tripp, R. A., Thierfelder, W. E., Witthuhn, B. A., McMickle, A. P., Doherty, P. C., Grosveld, G. C. & Ihle, J. N. (1995). Defective lymphoid development in mice lacking Jak3. *Science* **270**, 800-802.

Nunomura, S., Sato, T. & Habu, S. (2000). Molecular basis for functional maturation of thymocytes: increase in c-fos translation with positive selection. *The Journal of Immunology* **164**, 5590-5595.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**, 275-283.

Obrig, T. G., Culp, W. J., McKeehan, W. L. & Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *The Journal of Biological Chemistry* **246**, 174-181.

Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* **248**, 1517-1523.

Ohashi, P. S., Zinkernagel, R. M., Leuscher, I., Hengartner, H. & Pircher, H. (1993). Enhanced positive selection of a transgenic TCR by a restriction element that does not permit negative selection. *International Immunology* **5**, 131-138.

Ohoka, Y., Kuwata, T., Tozawa, Y., Zhao, Y., Mukai, M., Motegi, Y., Suzuki, R., Yokoyama, M. & Iwata, M. (1996). In vitro differentiation and commitment of CD4+ CD8+ thymocytes to the CD4 lineage, without TCR engagement. *International Immunology* **8**, 297-306.

Okayasu, I., Hatakeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y. & Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98**, 694-702.

Oukka, M., Ho, I. C., de la Brousse, F. C., Hoey, T., Grusby, M. J. & Glimcher, L. H. (1998). The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* **9**, 295-304.

Pai, S. Y., Truitt, M. L., Ting, C. N., Leiden, J. M., Glimcher, L. H. & Ho, I. C. (2003). Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity* **19**, 863-875.

Palacios, E. H. & Weiss, A. (2007). Distinct roles for Syk and ZAP-70 during early thymocyte development. *The Journal of Experimental Medicine* **204**, 1703-1715.

Pancer, Z., Amemiya, C. T., Ehrhardt, G. R., Ceitlin, J., Gartland, G. L. & Cooper, M. D. (2004). Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* **430**, 174-180.

Park, J. H., Yu, Q., Erman, B., Appelbaum, J. S., Montoya-Durango, D., Grimes, H. L. & Singer, A. (2004). Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* **21**, 289-302.

Park, J. H., Adoro, S., Lucas, P. J. & other authors (2007). 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. *Nature Immunology* **8**, 1049-1059.

Park, J. H., Adoro, S., Guinter, T. & other authors (2010). Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nature Immunology*.

Park, S. Y., Saijo, K., Takahashi, T. & other authors (1995). Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* **3**, 771-782.

Paterson, D. J. & Williams, A. F. (1987). An intermediate cell in thymocyte differentiation that expresses CD8 but not CD4 antigen. *The Journal of Experimental Medicine* **166**, 1603-1608.

Pellettieri, J. & Sanchez Alvarado, A. (2007). Cell turnover and adult tissue homeostasis: from humans to planarians. *Annual Reviews of Genetics* **41**, 83-105.

Penit, C., Vasseur, F. & Papiernik, M. (1988). In vivo dynamics of CD4-8- thymocytes. Proliferation, renewal and differentiation of different cell subsets studied by DNA biosynthetic labeling and surface antigen detection. *European Journal of Immunology* **18**, 1343-1350.

Penit, C. (1990). Positive selection is an early event in thymocyte differentiation: high TCR expression by cycling immature thymocytes precedes final maturation by several days. *International Immunology* **2**, 629-638.

Penit, C., Lucas, B. & Vasseur, F. (1995). Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *The Journal of Immunology* **154**, 5103-5113.

Peschon, J. J., Morrissey, P. J., Grabstein, K. H. & other authors (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *The Journal of Experimental Medicine* **180**, 1955-1960.

Philpott, K. L., Viney, J. L., Kay, G., Rastan, S., Gardiner, E. M., Chae, S., Hayday, A. C. & Owen, M. J. (1992). Lymphoid development in mice congenitally lacking T cell receptor alpha beta-expressing cells. *Science* **256**, 1448-1452.

Pilorget, A., Demeule, M., Barakat, S., Marvaldi, J., Luis, J. & Beliveau, R. (2007). Modulation of P-glycoprotein function by sphingosine kinase-1 in brain endothelial cells. *Journal of Neurochemistry* **100**, 1203-1210.

Ploegh, H. L., Orr, H. T. & Strominger, J. L. (1981). Major histocompatibility antigens: the human (HLA-A, -B, -C) and murine (H-2K, H-2D) class I molecules. *Cell* **24**, 287-299.

Polic, B., Kunkel, D., Scheffold, A. & Rajewsky, K. (2001). How alpha beta T cells deal with induced TCR alpha ablation. *Proceedings of the National Academy of Sciences U S A* **98**, 8744-8749.

Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W. & other authors (1991). Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* **353**, 180-184.

Ravichandran, K. S. & Burakoff, S. J. (1994). Evidence for differential intracellular signaling via CD4 and CD8 molecules. *The Journal of Experimental Medicine* **179**, 727-732.

Reich, E. (1964). Actinomycin: Correlation of Structure and Function of Its Complexes with Purines and DNA. *Science* **143**, 684-689.

Robey, E. A., Fowlkes, B. J., Gordon, J. W., Kioussis, D., von Boehmer, H., Ramsdell, F. & Axel, R. (1991). Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell* **64**, 99-107.

Rodewald, H. R. (2008). Thymus organogenesis. *Annual Review of Immunology* **26**, 355-388.

Rothenberg, E. V., Moore, J. E. & Yui, M. A. (2008). Launching the T-cell-lineage developmental programme. *Nature Reviews Immunology* **8**, 9-21.

Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L. & Schlossman, S. F. (1988). The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proceedings of the National Academy of Sciences U S A* **85**, 5190-5194.

Sadeghi-Shabestari, M., Vesal, S., Jabbarpour-Bonyadi, M., de Villatay, J. P., Fischer, A. & Rezaei, N. (2009). Novel RAG2 mutation in a patient with T- B- severe combined immunodeficiency and disseminated BCG disease. *Journal of Investigational Allergology and Clinical Immunology* **19**, 494-496.

Saini, M. (2007). The Role of TCR and Cytokine Signals in Naïve T cell Homeostasis. In *Immune Cell Biology*. London: National Institute for Medical Research.

Saini, M., Sinclair, C., Marshall, D., Tolaini, M., Sakaguchi, S. & Seddon, B. (2010). Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds. *Science Signaling* **3**, ra23.

Sakaguchi, N., Takahashi, T., Hata, H. & other authors (2003). Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* **426**, 454-460.

Sakaguchi, S., Fukuma, K., Kuribayashi, K. & Masuda, T. (1985). Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *The Journal of Experimental Medicine* **161**, 72-87.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology* **155**, 1151-1164.

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Reviews of Immunology* **22**, 531-562.

Sallusto, F., Geginat, J. & Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annual Reviews of Immunology* **22**, 745-763.

Samaridis, J., Casorati, G., Traunecker, A., Iglesias, A., Gutierrez, J. C., Muller, U. & Palacios, R. (1991). Development of lymphocytes in interleukin 7-transgenic mice. *European Journal of Immunology* **21**, 453-460.

Samelson, L. E. (2002). Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annual Reviews of Immunology* **20**, 371-394.

Sarafova, S. D., Erman, B., Yu, Q. & other authors (2005). Modulation of coreceptor transcription during positive selection dictates lineage fate independently of TCR/coreceptor specificity. *Immunity* **23**, 75-87.

Sarafova, S. D., Van Laethem, F., Adoro, S., Guinter, T., Sharrow, S. O., Feigenbaum, L. & Singer, A. (2009). Upregulation of CD4 Expression during MHC Class II-Specific Positive Selection Is Essential for Error-free Lineage Choice. *Immunity*.

Sato, T., Ohno, S., Hayashi, T., Sato, C., Kohu, K., Satake, M. & Habu, S. (2005). Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* **22**, 317-328.

Schatz, D. G., Oettinger, M. A. & Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. *Cell* **59**, 1035-1048.

Schatz, D. G., Oettinger, M. A. & Schlissel, M. S. (1992). V(D)J recombination: molecular biology and regulation. *Annual Reviews of Immunology* **10**, 359-383.

Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., Frew, E. & Scott, M. L. (2001). An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* **293**, 2111-2114.

Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrancois, L. (2000). Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature Immunology* **1**, 426-432.

Scollay, R. & Godfrey, D. I. (1995). Thymic emigration: conveyor belts or lucky dips? *Immunology Today* **16**, 268-273; discussion 273-264.

Scollay, R. G., Butcher, E. C. & Weissman, I. L. (1980). Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *European Journal of Immunology* **10**, 210-218.

Sebzda, E., Wallace, V. A., Mayer, J., Yeung, R. S., Mak, T. W. & Ohashi, P. S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science* **263**, 1615-1618.

Seddon, B., Legname, G., Tomlinson, P. & Zamoyska, R. (2000). Long-term survival but impaired homeostatic proliferation of Naive T cells in the absence of p56lck. *Science* **290**, 127-131.

Seddon, B. & Zamoyska, R. (2002a). TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. *The Journal of Immunology* **169**, 3752-3759.

Seddon, B. & Zamoyska, R. (2002b). TCR signals mediated by Src family kinases are essential for the survival of naive T cells. *The Journal of Immunology* **169**, 2997-3005.

Seddon, B., Tomlinson, P. & Zamoyska, R. (2003). Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nature Immunology* **4**, 680-686.

Seddon, B. & Zamoyska, R. (2003). Regulation of peripheral T-cell homeostasis by receptor signalling. *Current Opinion in Immunology* **15**, 321-324.

Seong, R. H., Chamberlain, J. W. & Parnes, J. R. (1992). Signal for T-cell differentiation to a CD4 cell lineage is delivered by CD4 transmembrane region and/or cytoplasmic tail. *Nature* **356**, 718-720.

Setoguchi, R., Tachibana, M., Naoe, Y., Muroi, S., Akiyama, K., Tezuka, C., Okuda, T. & Taniuchi, I. (2008). Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. *Science* **319**, 822-825.

Shaywitz, A. J. & Greenberg, M. E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual Review of Biochemistry* **68**, 821-861.

Sheng, M., Thompson, M. A. & Greenberg, M. E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Shinkai, Y., Rathbun, G., Lam, K. P. & other authors (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855-867.

Shores, E. W., Van Ewijk, W. & Singer, A. (1991). Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *European Journal of Immunology* **21**, 1657-1661.

Shortman, K., Vremec, D. & Egerton, M. (1991). The kinetics of T cell antigen receptor expression by subgroups of CD4+8+ thymocytes: delineation of CD4+8+3(2+) thymocytes as post-selection intermediates leading to mature T cells. *The Journal of Experimental Medicine* **173**, 323-332.

Siegel, R. S., Pandolfino, T., Guitart, J., Rosen, S. & Kuzel, T. M. (2000). Primary cutaneous T-cell lymphoma: review and current concepts. *Journal of Clinical Oncology* **18**, 2908-2925.

Singer, A., Adoro, S. & Park, J. H. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nature Reviews Immunology* **8**, 788-801.

Smith, C. A., Williams, G. T., Kingston, R., Jenkinson, E. J. & Owen, J. J. (1989). Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337**, 181-184.

Smith, K., Seddon, B., Purbhoo, M. A., Zamoyska, R., Fisher, A. G. & Merkenschlager, M. (2001). Sensory adaptation in naive peripheral CD4 T cells. *The Journal of Experimental Medicine* **194**, 1253-1261.

Smith, S. H. & Cancro, M. P. (2003). Cutting edge: B cell receptor signals regulate BLyS receptor levels in mature B cells and their immediate progenitors. *The Journal of Immunology* **170**, 5820-5823.

Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. (2009). T cell activation. *Annual Reviews of Immunology* **27**, 591-619.

Smyth, L. A., Williams, O., Huby, R. D., Norton, T., Acuto, O., Ley, S. C. & Kioussis, D. (1998). Altered peptide ligands induce quantitatively but not qualitatively different intracellular signals in primary thymocytes. *Proceedings of the National Academy of Sciences U S A* **95**, 8193-8198.

Sobacchi, C., Marrella, V., Rucci, F., Vezzoni, P. & Villa, A. (2006). RAG-dependent primary immunodeficiencies. *Human Mutation* **27**, 1174-1184.

Sprent, J. & Tough, D. F. (1994). Lymphocyte life-span and memory. *Science* **265**, 1395-1400.

Stadanlick, J. E. & Cancro, M. P. (2008). BAFF and the plasticity of peripheral B cell tolerance. *Current Opinion in Immunology* **20**, 158-161.

Stadanlick, J. E., Kaileh, M., Karnell, F. G. & other authors (2008). Tonic B cell antigen receptor signals supply an NF-kappaB substrate for prosurvival BLyS signaling. *Nature Immunology* **9**, 1379-1387.

Starr, T. K., Jameson, S. C. & Hogquist, K. A. (2003). Positive and negative selection of T cells. *Annual Reviews of Immunology* **21**, 139-176.

Steen, A. M., Luthman, H., Hellgren, D. & Lambert, B. (1990). Levels of hypoxanthine phosphoribosyltransferase RNA in human cells. *Experimental Cell Research* **186**, 236-244.

Stefanova, I., Dorfman, J. R. & Germain, R. N. (2002). Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. *Nature* **420**, 429-434.

Stutman, O. (1986). Postthymic T-cell development. *Immunological Reviews* **91**, 159-194.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M. & Yoshida, H. (1993). Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proceedings of the National Academy of Sciences U S A* **90**, 9125-9129.

Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T. & Hidaka, H. (1991). The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochemical and Biophysical Research Communications* **181**, 968-975.

Sun, G., Liu, X., Mercado, P., Jenkinson, S. R., Kypriotou, M., Feigenbaum, L., Galera, P. & Bosselut, R. (2005). The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nature Immunology* **6**, 373-381.

Surh, C. D., Ernst, B. & Sprent, J. (1992). Growth of epithelial cells in the thymic medulla is under the control of mature T cells. *The Journal of Experimental Medicine* **176**, 611-616.

Surh, C. D. & Sprent, J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372**, 100-103.

Surh, C. D. & Sprent, J. (2008). Homeostasis of naive and memory T cells. *Immunity* **29**, 848-862.

Swat, W., Dessing, M., von Boehmer, H. & Kisielow, P. (1993). CD69 expression during selection and maturation of CD4+8+ thymocytes. *European Journal of Immunology* **23**, 739-746.

Takada, K. & Jameson, S. C. (2009). Self-class I MHC molecules support survival of naive CD8 T cells, but depress their functional sensitivity through regulation of CD8 expression levels. *The Journal of Experimental Medicine* **206**, 2253-2269.

Takeda, K., Kaisho, T. & Akira, S. (2003). Toll-like receptors. *Annual Reviews of Immunology* **21**, 335-376.

Tan, J. T., Dudl, E., LeRoy, E., Murray, R., Sprent, J., Weinberg, K. I. & Surh, C. D. (2001). IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proceedings of the National Academy of Sciences U S A* **98**, 8732-8737.

Tanchot, C. & Rocha, B. (1995). The peripheral T cell repertoire: independent homeostatic regulation of virgin and activated CD8+ T cell pools. *European Journal of Immunology* **25**, 2127-2136.

Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y. & Littman, D. R. (2002). Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111**, 621-633.

Taniuchi, I. & Littman, D. R. (2004). Epigenetic gene silencing by Runx proteins. *Oncogene* **23**, 4341-4345.

Tarakhovsky, A., Kanner, S. B., Hombach, J., Ledbetter, J. A., Muller, W., Killeen, N. & Rajewsky, K. (1995). A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* **269**, 535-537.

Thompson, J. S., Bixler, S. A., Qian, F. & other authors (2001). BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* **293**, 2108-2111.

Tough, D. F. & Sprent, J. (1994). Turnover of naive- and memory-phenotype T cells. *The Journal of Experimental Medicine* **179**, 1127-1135.

Ueno, T., Hara, K., Willis, M. S. & other authors (2002). Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity* **16**, 205-218.

Ueno, T., Saito, F., Gray, D. H. & other authors (2004). CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *The Journal of Experimental Medicine* **200**, 493-505.

Van De Wiele, C. J., Marino, J. H., Murray, B. W., Vo, S. S., Whetsell, M. E. & Teague, T. K. (2004). Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. *The Journal of Immunology* **172**, 4235-4244.

van Ewijk, W. (1991). T-cell differentiation is influenced by thymic microenvironments. *Annual Reviews of Immunology* **9**, 591-615.

van Lookeren Campagne, M., Wiesmann, C. & Brown, E. J. (2007). Macrophage complement receptors and pathogen clearance. *Cellular Microbiology* **9**, 2095-2102.

Vasseur, F., Le Campion, A. & Penit, C. (2001). Scheduled kinetics of cell proliferation and phenotypic changes during immature thymocyte generation. *European Journal of Immunology* **31**, 3038-3047.

Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* **55**, 301-308.

Venkitaraman, A. R. & Cowling, R. J. (1994). Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. *European Journal of Immunology* **24**, 2168-2174.

Vig, M. & Kinet, J. P. (2009). Calcium signaling in immune cells. *Nature Immunology* **10**, 21-27.

Villa, A., Sobacchi, C., Notarangelo, L. D. & other authors (2001). V(D)J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. *Blood* **97**, 81-88.

Viret, C., Wong, F. S. & Janeway, C. A., Jr. (1999). Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. *Immunity* **10**, 559-568.

Vivien, L., Benoist, C. & Mathis, D. (2001). T lymphocytes need IL-7 but not IL-4 or IL-6 to survive in vivo. *International Immunology* **13**, 763-768.

von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. & Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *The Journal of Experimental Medicine* **181**, 1519-1526.

von Freeden-Jeffry, U., Solvason, N., Howard, M. & Murray, R. (1997). The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity* **7**, 147-154.

Vyse, T. J. & Todd, J. A. (1996). Genetic analysis of autoimmune disease. *Cell* **85**, 311-318.

Walmsley, M. J., Ooi, S. K., Reynolds, L. F. & other authors (2003). Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. *Science* **302**, 459-462.

Wang, L., Wildt, K. F., Zhu, J., Zhang, X., Feigenbaum, L., Tessarollo, L., Paul, W. E., Fowlkes, B. J. & Bosselut, R. (2008). Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. *Nature Immunology* **9**, 1122-1130.

Wang, Q., Strong, J. & Killeen, N. (2001). Homeostatic competition among T cells revealed by conditional inactivation of the mouse Cd4 gene. *The Journal of Experimental Medicine* **194**, 1721-1730.

Wange, R. L., Malek, S. N., Desiderio, S. & Samelson, L. E. (1993). Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor zeta and CD3 epsilon from activated Jurkat T cells. *The Journal of Biological Chemistry* **268**, 19797-19801.

Wardenburg, J. B., Fu, C., Jackman, J. K. & other authors (1996). Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *The Journal of Biological Chemistry* **271**, 19641-19644.

Watts, C. (1997). Capture and processing of exogenous antigens for presentation on MHC molecules. *Annual Reviews of Immunology* **15**, 821-850.

Weerkamp, F., Pike-Overzet, K. & Staal, F. J. (2006). T-sing progenitors to commit. *Trends in Immunology* **27**, 125-131.

Weinreich, M. A. & Hogquist, K. A. (2008). Thymic emigration: when and how T cells leave home. *The Journal of Immunology* **181**, 2265-2270.

Wiest, D. L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R. D., Glimcher, L. H., Samelson, L. E. & Singer, A. (1993). Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *The Journal of Experimental Medicine* **178**, 1701-1712.

Wildin, R. S., Smyk-Pearson, S. & Filipovich, A. H. (2002). Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *Journal of Medical Genetics* **39**, 537-545.

Wildt, K. F., Sun, G., Grueter, B., Fischer, M., Zamisch, M., Ehlers, M. & Bosselut, R. (2007). The transcription factor Zbtb7b promotes CD4 expression by antagonizing Runx-mediated activation of the CD4 silencer. *The Journal of Immunology* **179**, 4405-4414.

Williams, L. M. & Rudensky, A. Y. (2007). Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature Immunology* **8**, 277-284.

Wilson, A., Day, L. M., Scollay, R. & Shortman, K. (1988). Subpopulations of mature murine thymocytes: properties of CD4-CD8+ and CD4+CD8-thymocytes lacking the heat-stable antigen. *Cellular Immunology* **117**, 312-326.

Witherden, D., van Oers, N., Waltzinger, C., Weiss, A., Benoist, C. & Mathis, D. (2000). Tetracycline-controllable selection of CD4(+) T cells: half-life and survival signals in the absence of major histocompatibility complex class II molecules. *The Journal of Experimental Medicine* **191**, 355-364.

Witt, C. M., Raychaudhuri, S., Schaefer, B., Chakraborty, A. K. & Robey, E. A. (2005). Directed migration of positively selected thymocytes visualized in real time. *PLoS Biology* **3**, e160.

Wong, P., Goldrath, A. W. & Rudensky, A. Y. (2000). Competition for specific intrathymic ligands limits positive selection in a TCR transgenic model of CD4+ T cell development. *The Journal of Immunology* **164**, 6252-6259.

Wong, P. & Pamer, E. G. (2003). CD8 T cell responses to infectious pathogens. *Annual Reviews of Immunology* **21**, 29-70.

Woolf, E., Xiao, C., Fainaru, O. & other authors (2003). Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. *Proceedings of the National Academy of Sciences U S A* **100**, 7731-7736.

Xue, H. H., Bollenbacher, J., Rovella, V., Tripuraneni, R., Du, Y. B., Liu, C. Y., Williams, A., McCoy, J. P. & Leonard, W. J. (2004). GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells. *Nature Immunology* **5**, 1036-1044.

Yablonski, D., Kuhne, M. R., Kadlecek, T. & Weiss, A. (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. *Science* **281**, 413-416.

Yablonski, D., Kadlecek, T. & Weiss, A. (2001). Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. *Molecular and Cellular Biology* **21**, 4208-4218.

Yang, Y., Contag, C. H., Felsher, D., Shachaf, C. M., Cao, Y., Herzenberg, L. A. & Tung, J. W. (2004). The E47 transcription factor negatively regulates CD5 expression during thymocyte development. *Proceedings of the National Academy of Sciences U S A* **101**, 3898-3902.

Yao, Z., Cui, Y., Watford, W. T. & other authors (2006). Stat5a/b are essential for normal lymphoid development and differentiation. *Proceedings of the National Academy of Sciences U S A* **103**, 1000-1005.

Yasutomo, K., Doyle, C., Miele, L., Fuchs, C. & Germain, R. N. (2000). The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. *Nature* **404**, 506-510.

Yates, A., Saini, M., Mathiot, A. & Seddon, B. (2008). Mathematical modeling reveals the biological program regulating lymphopenia-induced proliferation. *The Journal of Immunology* **180**, 1414-1422.

Yeganeh, M., Heidarzade, M., Pourpak, Z. & other authors (2008). Severe combined immunodeficiency: a cohort of 40 patients. *Pediatric Allergy and Immunology* **19**, 303-306.

Yin, X., Chtanova, T., Ladi, E. & Robey, E. A. (2006). Thymocyte motility: mutants, movies and migration patterns. *Current Opinion in Immunology* **18**, 191-197.

York, I. A. & Rock, K. L. (1996). Antigen processing and presentation by the class I major histocompatibility complex. *Annual Reviews of Immunology* **14**, 369-396.

Yoshida, R., Imai, T., Hieshima, K. & other authors (1997). Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *The Journal of Biological Chemistry* **272**, 13803-13809.

Yu, Q., Erman, B., Bhandoola, A., Sharrow, S. O. & Singer, A. (2003). In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. *The Journal of Experimental Medicine* **197**, 475-487.

Yu, Q., Park, J. H., Doan, L. L., Erman, B., Feigenbaum, L. & Singer, A. (2006). Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *The Journal of Experimental Medicine* **203**, 165-175.

Zachariah, M. A. & Cyster, J. G. (2010). Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction. *Science* **328**, 1129-1135.

Zeng, R., Cannon, J. L., Abraham, R. T., Way, M., Billadeau, D. D., Bubeck-Wardenberg, J. & Burkhardt, J. K. (2003). SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *The Journal of Immunology* **171**, 1360-1368.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P. & Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92**, 83-92.

Zhu, J., Yamane, H. & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). *Annual Reviews of Immunology* **28**, 445-489.

Zhumabekov, T., Corbella, P., Tolaini, M. & Kioussis, D. (1995). Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *The Journal of Immunological Methods* **185**, 133-140.

Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Raulet, D. H. & Jaenisch, R. (1990). Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature* **344**, 742-746.

Zinkernagel, R. M. & Doherty, P. C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702.

Appendix

Saini, M.*, Sinclair, C.*, Marshall, D., Tolaini, M., Sakaguchi, S. & Seddon, B. (2010). Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds. *Science Signaling* **3**, ra23. *****Contributed equally.

Available in print version or online (see below).

Regulation of Zap70 Expression During Thymocyte Development Enables Temporal Separation of CD4 and CD8 Repertoire Selection at Different Signaling Thresholds

Manoj Saini, Charles Sinclair, Daniel Marshall, Mauro Tolaini, Shimon Sakaguchi and Benedict Seddon (23 March 2010)

Science Signaling **3** (114), ra23. [DOI: 10.1126/scisignal.2000702]